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Developmental variations in environmental influences including endocrine disruptors on pubertal timing and neuroendocrine control: Revision of human observations and mechanistic insight from rodents

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ABSTRACT

Puberty presents remarkable individual differences in timing reaching over 5 years in humans. We put emphasis on the two edges of the age distribution of pubertal signs in humans and point to an extended distribution towards earliness for initial pubertal stages and towards lateness for final pubertal stages. Such distortion of distribution is a recent phenomenon. This suggests changing environmental influences including the possible role of nutrition, stress and endocrine disruptors. Our ability to assess neuroendocrine effects and mechanisms is very limited in humans. Using the rodent as a model, we examine the impact of environmental factors on the individual variations in pubertal timing and the possible underlying mechanisms. The capacity of environmental factors to shape functioning of the neuroendocrine system is thought to be maximal during fetal and early postnatal life and possibly less important when approaching the time of onset of puberty. © 2014 Elsevier Inc. All rights reserved.

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1. Introduction

1.1. The classical paradigm of secular advance in human pubertal timing

Fig. 1 summarizes the data on changes in menarcheal age that are used classically to illustrate the secular trend in pubertal timing in various countries. Environmental factors have been thought to account for the reduction in menarcheal age that has been reported since 1850 till 1960 in Scandinavian countries (Tanner, 1962) and further in many European countries and USA (reviewed in Parent et al. (2003)). These findings were interpreted as a result of the improvement in life standards and socio-economical conditions (Biro et al., 2006; Dunger et al., 2006; Ong et al., 2004, 2006; Cheng et al., 2012; Himes, 2006; Roa and Tena-Sempere, 2010). A projection after 1960 of the former reduction seen in Scandinavian countries (Fig. 1) indicates that a sustained secular trend would have led to a mean menarcheal age of less than 12 yrs by the end of the 20th century. This was not the case: after 1960, the secular advancement in female pubertal timing has become less rapid or has even come to an end in countries such as Sweden, Belgium and Hungary (reviewed in Parent et al. (2003)) (Fig. 1). However, menarcheal age has shown a very rapid progression in countries like China and India (reviewed in Parent et al. (2003)) where the standard of life has improved recently. Altogether, those data are consistent with a prominent role of nutrition availability. The "critical weight/fat mass" theory proposed by Frisch and Revelle (1970) and subsequent work (Biro et al., 2006; Dunger et al., 2006; Ong et al., 2004, 2006; Cheng et al., 2012; Himes, 2006; Roa and Tena-Sempere, 2010) have put emphasis on the role of nutritional conditions based on adiposity in puberty, at the time of menarche. The discovery of leptin (Zhang et al., 1994; Campfield et al., 1995) and its prerequisite role in the neuroendocrine control of pubertal maturation and reproduction (reviewed in Sanchez-Garrido and Tena-Sempere (2013)) has added to the importance of energy balance in the prepubertal period to enable onset and progression of puberty.

The secular advancement in pubertal timing has been established following observations about mean or median age at menarche. Implicitly, the whole female pubertal process was thought to undergo similar changes though there were no data available to confirm that. The existence of similar changes for male puberty remains putative. Recent study (Goldstein, 2011) has investigated the secular trend in age at increased mortality in males, assuming that mortality at that age is due to adolescent risk taking behaviors presumably depending on pubertal timing. Because that male adolescent mortality hump fell from an average of 21 years in 1850 to 18 years in 1960, the author concluded to a likely secular advancement in pubertal age in males. Few decades ago, the levelling off or arrested secular reduction in average menarcheal age led to the conclusion that stabilization had occurred after resetting pubertal timing to younger ages. Such a conclusion had implications on issues raised by scientists and clinicians: no further changes in pubertal timing could mean that environmental factors were stable

and that the previously defined age limits for pubertal disorders were still valid. In 1961, Thamdrup (1961) proposed the age limits of 8 years and 9 years for diagnosis of sexual precocity in girls and boys respectively. Fifty years later, those age limits have not been revised, so far, though as discussed below, the age limits for onset of puberty in the population of many countries have changed in the recent past.

1.2. Pubertal timing and preceding life periods across species

Pubertal neuroendocrine activation or reactivation of the pitui-tary-gonadal axis is essential for achievement of reproductive capacity. A leading factor in that process is Gonadotropin Releasing Hormone (GnRH) that is released by median eminence terminals of peptidergic neurons in a pulsatile manner showing increased frequency and amplitude at the onset of puberty (Grumbach, 2002; Terasawa and Fernandez, 2001; Plant, 2008; Lomniczi et al., 2013; Ojeda and Lomniczi, 2014). This event occurs at a time in life that varies both among species and within a single species. In Fig. 2 are shown the species-related differences in relative duration of the prepubertal latency (from birth to puberty) when calculated as a percentage of lifespan for comparison purposes. Ewe (Foster et al., 1985), rat (Maeda et al., 2000) and quail (Ottinger et al., 2003) start puberty after a latency accounting for 4.8-5.7% of the lifespan as opposed to 16.3% in humans (Roelants et al., 2009) and 22.5% in baboons (Onyango et al., 2013) that is about 3-4 times longer than in non-primate species. Another less emphasized species-related difference is the variance of pubertal timing among individuals. The timing of puberty shows important differences between human individuals and the physiological range (3rd to 97th centile) of 4.8 years (Roelants et al., 2009) represents 6.25% of the life span. In the laboratory rat with a life expectancy of 2 years, the timing of puberty varies within 4-5 days (Maeda et al., 2000) accounting for an individual variance of 0.55% which is 11 times less than in humans. In sheep (Foster et al., 1985), and quail (Ottinger et al., 2003), the variance of pubertal timing represents 0.87% and 1.9% of lifespan, respectively, that is also less than in humans (Fig. 2). The baboon, a subhuman primate shows a variance of pubertal timing (Onyango et al., 2013) that is even longer than in humans, when expressed relatively to average lifespan (10.0%). These data indicate that inter-individual variations in pubertal timing may be influenced by evolution across species. These data also suggest that not only the timing i.e. the latency between birth and mean or median age at a given pubertal sign is worth being studied but also the variance i.e. the time period between the earliest and latest individuals in a reference population for occurrence of a given pubertal sign. Both parameters (latency and variance) are likely influenced by environmental factors and could even be differentially affected with different mechanisms possibly involved. In the present paper, we will review comparatively the impact of different environmental factors on pubertal timing in humans and in animal models. The variance of pubertal timing has a different magnitude across species (Fig. 2) and may not have the same significance in animals and in humans. Laboratory animals are more homogeneous in term of genetic background and are exposed to a very regulated environment. However, they are unavoidable models when it comes to study the mechanisms of neuroendocrine regulation of pubertal timing. Since some data obtained in human, non-human primates and rodents will be discussed with emphasis on neuroendocrine maturation, it is important to keep in mind that when birth takes place in rodents, maturation of the brain is less advanced than in human newborns (Clancy et al., 2007). In the rat, the onset of puberty is marked by an increase in testicular weight increase in males and vaginal opening followed by the first estrus in female rodents and (Maeda et al., 2000). The onset of puberty takes place around the time of weaning by the age of 3 weeks as evidenced from onset of increase in serum levels of gonadal hormones (Maeda et al., 2000). It is preceded by attainment of a pubertal pattern of pulsatile GnRH secretion from hypothalamic explants (Bourguignon and Franchimont, 1984; Bourguignon et al., 1990, 1992). In humans, puberty is characterized by an increase in LH and gonadal hormones. In boys, puberty is marked by an increase in testicular volume above 3 ml, consistent with Tanner G2 stage (Parent et al., 2003). In girls, the earliest manifestation of puberty is acceleration in growth velocity but commonly used markers of the timing of female puberty are the first appearance of breast development defined as Tanner B2 stage followed by menarche (Parent et al., 2003). Table 1 summarizes the different developmental stages in humans and rats.

Fig. 1. Evolution of average menarcheal age (year) in the USA and Nordic countries between 1890 and 1960 (data compiled by Tanner (1962) and further, between 1960 and 2010, in different countries in Europe, USA and around the world (updated data compiled by Parent et al. (2003)). The broken red line represents the projected reduction after 1960, based on the former changes in Scandinavian countries as reported by Tanner: mean menarcheal age would have fallen down to below 12 yrs by the end of the 20th century. In fact, after 1960, average menarcheal age has leveled off in many countries while still progressing rapidly in countries such as India or China.

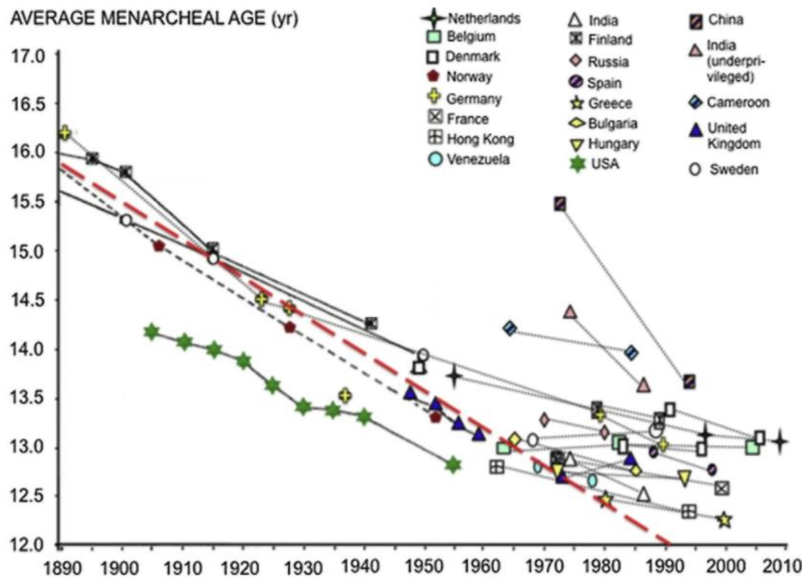


Fig. 2. The length of the prepubertal latency from birth to puberty (A) and the variance of pubertal timing (B) are represented as a percentage of lifespan in females of different species. Timing of puberty is defined here by median age at 1st ovulation. Variance of pubertal timing is calculated using the difference between upper and lower age limits for occurrence of female cycling.

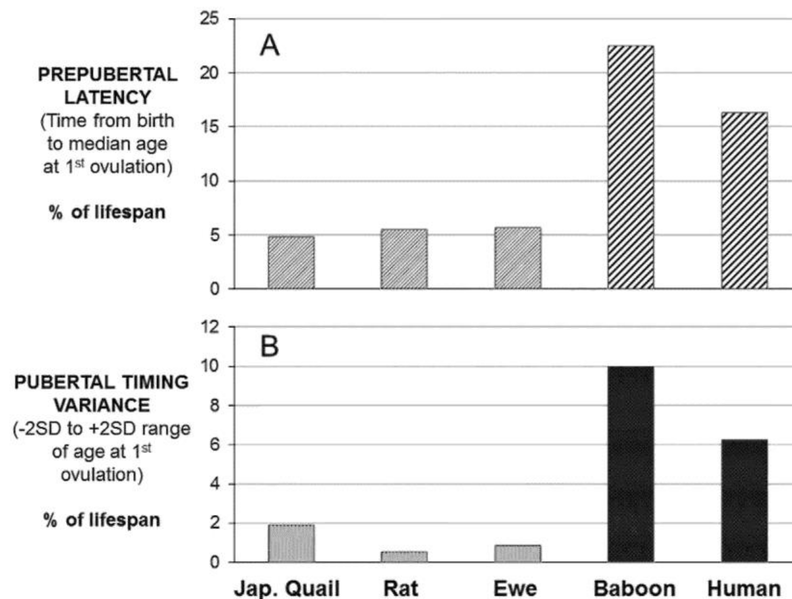


Table 1: Average time limits for definition of stages of human reproductive development and approximation to developmental stages in the laboratory rats.

Period	Fetal	Neonatal	Infantile	Juvenile/prepubertal	Pubertal	Post-pubertal
Time limits	Before birth	Soon after birth	Birth - end of lactation	End of lactation - onset of puberty	Onset[a] - end of puberty[b]	After end of puberty
Female rat	Gestational day 1-21.5		PN day 1-20.5	PN day 20.5-33	PN day 33-42	>42 days
Female human	Gestational day 1-280	PN month 0-1	PN month 1-18	PN year 1.5-11	PN year 11-14 yrs	>14 yrs

[a] Vaginal opening in rats and onset of breast development in girls. [b] First estrus in rats and menarche in girls.

1.3. Genetic regulation of pubertal timing and environmental interactions

The activation or reactivation of GnRH secretion at puberty is regulated by a complex neuronal and glial network within the hypothalamus. The synaptic changes leading to activation of GnRH secretion involve an increase of direct excitatory input through neurons such as kisseptin and glutamate neurons and a decrease of inhibitory input from neurons such as GABA neurons (Grumbach, 2002; Terasawa and Fernandez, 2001; Plant, 2008). In addition, glial cells play a facilitatory role through production of growth factors and small molecules (Ojeda, 2010). As suggested by the complexity of this network, regulation of puberty involves redundant pathways. However, some monogenic mutations such as those affecting the GnRH receptor, Kiss1 or Kisspeptin receptor (GPR54), TAC3 or TACR3 receptor (de Roux et al., 2003; Topaloglu et al., 2010) lead to puberty failure. While mutations of those genes are associated with absence of puberty, several studies have shown that variants of more than 30 genes are associated with age at menarche in humans (Elks et al., 2010). Amongst those genes, LIN28B and the 9q31.2 locus have been identified as associated with age at menarche in several independent genomewide studies (He et al., 2009; Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009). Such genes, however, are supposed to explain very few (<1%) of the variance in age at menarche (He et al., 2009). Many of the other identified loci are associated with BMI or energy homeostasis (Elks et al., 2010). While GPR54 (Teles et al., 2008; Leka-Emiri et al., 2014), TACR3 (Leka-Emiri et al., 2014) and LIN28B (Silveira-Neto et al., 2012) mutations seem to be very uncommon or absent in patients with precocious puberty (PP), inactivating mutations in the makorin ring finger protein 3 (*MKRN3*) gene, have been recently identified in 2-3% of sporadic PP and even more frequently in familial PP (Abreu et al., 2013; Macedo et al., 2014; Schreiner et al., 2014) and result in invalidation of a so far unidentified inhibitory neuroendocrine mechanism.

Genetic factors are considered to explain 50-80% of the variance of age at puberty (Wehkalampi et al., 2008). Recent studies have started to unravel the possible links between environment and the genetic control of pubertal timing where epigenetic mechanisms can play an important role. Lomniczi et al. have shown that *Kiss 1* expression is negatively regulated by a protein complex, the polycomb group (Ojeda and Lomniczi, 2014). At the initiation of puberty, DNA methylation of two members of this complex (*Eed* and *Cbx7*) increases, leading to a decrease of their expression and a decreased association of their proteic products with the *Kiss1* promoter. Moreover, inhibition of DNA methylation prevents the peripubertal decrease of *Eed* and *Cbx7* and their removal from the *Kiss1* promoter which results in puberty failure. These recent discoveries underline the crucial role of epigenetic mechanisms in the regulation of puberty and shed a new light on the possible effects of environmental factors on those epigenetic mechanisms. The effects of the environment on the hypothalamic epigenome have not been unraveled yet but several studies have shown that the adolescent brain epigenome is affected by environmental perturbations (Morrison et al., 2013). For instance, exposure of pubertal male rats to cannabioid leads to long-term changes in gene *H3K9* methylation thereby dysregulating the proenkephalin system in the nucleus accumbens (Tomasiewicz et al., 2012). Pubertal exposure to alcohol has been shown to lead to long-term changes in histone acetylation and related gene expression in the rat prefrontal cortex (Pascual et al., 2012). Thus, while recent data have shown epigenetic regulation of puberty at the hypothalamic level, the epigenetic effects of environmental factors on the hypothalamic regulation of puberty are still to be discovered.

Genome-wide studies have shown that Lin 28 is associated with age at puberty (Ong et al., 2009). Lin 28 is a RNA-binding protein that inhibits the maturation of miRNA of the Let family. Some data suggest a role of the

Lin28/Let-7 system in the hypothalamic control of puberty (Sangiao-Alvarellos et al., 2013). In male and female rats, *Lin28, Lin28b,* and *c-Myc* mRNAs (upstream positive regulator of *Lin28*) display very high hypothalamic expression during the neonatal period, markedly decreased during the infantile-to-juvenile transition and reach minimal levels before/around puberty in rats and monkeys. Conversely, *let-7a, let-7b, mir-132,* and *mir-145* show opposite expression profiles (Sangiao-Alvarellos et al., 2013). In addition, manipulations disturbing puberty such as delayed puberty induced by either neonatal exposure to sex steroids or infantile exposure to constant darkness hamper these changes, indicating that the Lin28/Let-7 system could mediate some of the effects of environment on puberty. In contrast, altered puberty due to manipulation of caloric intake before weaning or between weaning and puberty does not result in comparable changes.

The data summarized in the previous paragraph suggest that the peripubertal period could be particularly sensitive to the epigenetic effects of the environment on the hypothalamic regulation of puberty. It appears that the environment also affects epigenetic pathways much earlier during development and can lead to predisposition to some disease later in life. For instance, *in vitro* and *in vivo* models have established that epigenetic modifications caused by *in utero* exposure to endocrine disrupting chemicals (EDCs) can induce alterations in gene expression that may persist throughout life. Using heterozygous yellow agouti mice (A^{vy}) in which the *agouti* gene encodes a molecule that promotes follicular melanocytes to produce yellow pigment instead of black, Dolinoy et al. showed that maternal exposure to environmentally relevant doses of BPA resulted in altered DNA methylation at 2 metastable loci leading to a change of fur color. Restoration of normal methylation patterns occurred with maternal supplementation of geni-stein or methyl donors (Dolinoy et al., 2007). Maternal exposure to phthalates, another group of anti-androgenic plasticizers, has been shown to increase DNA methylation and DNA methyltrans-ferases expression in mouse testis (Wu et al., 2010). Given the sensitivity of the organism to alteration of methylation early in life, one can hypothesize that early exposure to alteration of the epigenome could lead to perturbation of the hypothalamic control of puberty later in life.

1.4. Dual critical periods (fetal/neonatal life vs prepuberty) for environmental effects

In the following sections, we will discuss how the developmental stage can influence the effects of several factors (energy availability through nutrition, stress, sex steroids, endocrine disruptors) on pubertal timing. Two critical periods will be considered. Classically, the intrauterine and neonatal periods are considered to be essential for sexual differentiation and the prepubertal period is essential for sexual maturation. Beyond this effect, Barker's observations (Barker and Osmond, 1986) led to the concept of fetal set-up of lifelong adaptive mechanisms (known as the developmental origin of health and disease) that can be relevant to the neuroendocrine control of the onset of puberty: programming of the timing of sexual maturation as an adaptive mechanism under the early influence of environmental factors.

The effects of environment are variable, depending on several factors. For instance, prepubertal restriction or excess of food and adiposity may lead to delayed or early puberty, respectively (Biro et al., 2006; Dunger et al., 2006; Ong et al., 2004, 2006; Cheng et al., 2012; Himes, 2006; Roa and Tena-Sempere, 2010) whereas intrauterine growth restriction is associated with early puberty (Ibanez et al., 2011, 2006) in humans. Similarly, psychosocial stress during prepuberty or puberty may cause pubertal delay or arrest (van Noord and Kaaks, 1991; Tahirovic, 1998) whereas advancement of puberty has been described in children who had experienced such stress in early postnatal life or infancy (Moffitt et al., 1992; Wierson et al., 1993). In a finalist and maybe simplistic interpretation, the data discussed in this review provide evidence that environmental clues may affect pubertal timing differently depending on the life period when they come into action. In the early phase of organization of homeostasis i.e. adaptive mechanisms, adverse conditions can be interpreted by the hypothalamic centers as a risk for species survival and are translated into need of early puberty and reproduction. Reversely, in a period closer to puberty, similar adverse conditions can be interpreted as a risk for quality and outcome of pregnancy and are translated into need of delayed puberty and reproduction. Along that interpretation, the advancement of puberty in children migrating for international adoption could result from a combination of early life adversity and late prepubertal life opulence, two opposing conditions favoring advancement of puberty. Such a concept is consistent with life history interpretation of the control of reproduction that is based upon interaction between intrinsic and extrinsic constraints as reviewed and discussed by Sloboda et al. (2011).

1.5. Issues for revision: endpoints of pubertal timing and mechanisms of environmental effects in relation to period of exposure

The classical paradigm of environmental effects on pubertal development lies on secular changes in mean or median age at a particular event of puberty such as menarche. We will review and describe detailed characteristics of the changes in pubertal timing possibly involving different signs and events in the sequence of pubertal maturation. Our purpose is to discuss the impact of environmental factors in that process including the putative role of endocrine disruptors with emphasis on the life period when they take place and the possible neuroendocrine mechanisms.

2. Variable pattern of pubertal timing depending on pubertal endpoint

Though the mean or median age at occurrence of a given pubertal sign has been used as the sole indicator of pubertal timing in many studies, detailed analysis shows that more subtle changes occur based on the pattern of age distribution both in boys and girls. Moreover, in clinical practice, the extreme lower and upper age limits in the normal population provide important information since they are used to define early or late maturation. The distance i.e. the age difference can be calculated between the median age at occurrence of a given pubertal sign and either the 3rd or 10th cen-tile on the one hand and the 97th or 90th centile on the other hand. Such an age distance is equal on both sides of the median when the distribution is normal or Gaussian and then only, standard deviations allow determination of the normal age limits. In case of unequal (asymmetrical) distribution, the difference between the distances (median age - lower limit) and (median age - upper limit) provides an appraisal of distortion that is negative when deviation is towards younger ages and positive when it is towards older ages. Such a calculation is illustrated (Fig. 3) using the Belgian data published recently (Roelants et al., 2009). In boys, the data are represented through both the cumulative frequency (Fig. 3A) or the absolute frequency (Fig. 3B) of ages when achieving testicular volume ≥4ml and ≥ 15ml that are respectively consistent with onset and final stage of puberty. The asymmetry of the curves is obvious. In comparison with the theoretical curve of a normal distribution (Fig. 3B), the two age distribution curves are opposed by a distortion that is negative and positive for 4 and 15 ml testicular volume, respectively. These data indicate that currently, some boys tend to enter puberty earlier and some to end puberty later. In girls (Fig. 3C), the current curve of cumulative frequency for occurrence of breast (B2) seems to parallel that for menarche. Calculation of distortion at centile 3 and 97, however, reveals a negative value for B2, i.e. distribution skewed towards earliness and a positive value for menarche, i.e. distribution skewed towards lateness. Literature data on median age and upper/lower limits for occurrence of pubertal signs in boys and girls are compiled in Table 2. Using those data, we have calculated the distortion of variance at the 10th and 90th centiles of age. When studying distribution of data from 95% of the population i.e. centile 3rd to 97th (Roelants et al., 2009) such as shown in Fig. 3, it is of note that distortion is twice as much as it is using data from 80% of the population i.e. centile 10th to 90th (Table 2). This difference exists because the distortion of variance is all the more important since the extreme tail of the distribution is included. However, 3rd and 97th centile data were rarely provided in the publications and therefore calculation of distortion in Table 2 is based on 10th and 90th centile data.

Among women born in Belgium in the 1920s (Jeurissen, 1969), the median menarcheal age was 1.5 years later than in more recent cohorts and variance (10th to 90th centiles) was 4.1 years. The distortion of variance in age at menarche was negative at that time. In a Belgian cohort born in the 1960s (Vercauteren and Susanne, 1985), the median menarcheal age was advanced to 13yrs. The distance between the 10th and the 90th centile had fallen to 2.4 yrs indicating that the variance was decreasing and the distortion was still negative. In the most recent Belgian cohort born in the 1990s (Roelants et al., 2009), the median menarcheal age did no longer change while the distance between the 10th and the 90th centiles had increased to 3.1 yrs indicating that the variance was increasing and distortion became positive. Among the different countries, except the data obtained before the secular trend in menarcheal age was leveling off (van Noord and Kaaks, 1991), there were only minor differences both in the median menarcheal age that varied between 13.0 and 13.4 yrs and in the variance i.e. the 10th to 90th centile range (Table 2). Distortion of the variance was negative in the studies involving subjects born before the 1970s (Jeurissen, 1969; Vercauteren and Susanne, 1985) and was found to be positive in several more recent studies since 1980 (Roelants et al., 2009; Talma et al., 2013; Mul et al., 2001). Consequently (Fig. 4A), there is a secular shift in distortion of variance in menarcheal age from negative to positive values during the 20th century. These data are consistent with a French report of a secular increase in proportion of women needing 5 years after menarche before attainment of regular (presumably ovulatory) cycling while there was concomitantly a secular reduction in the proportion getting menarche at 15 years or later (Clavel-Chapelon and E3N-EPIC group, 2002).

The median age at onset of breast development appears to be earlier in USA, Denmark and Greece (Aksglaede et al., 2009; Herman-Giddens et al., 1997; Papadimitriou et al., 2008) than in the Netherlands and Belgium (Roelants et al., 2009; Mul et al., 2001). The distance between the 10th and the 90th centile (Table 2) is quite similar among the studies and a slight negative distortion of variance is reported in 3 studies (Roelants et al., 2009; Mul et al., 2001; Papadimitriou et al., 2008). As opposed to the secular trend in distortion of variance in menarcheal age, the data on age at onset of breast development in several countries show a negative distortion that seems to be more marked in the recent studies (Fig. 4A). Taken together, those observations indicate that, during the last decades of the 20th century, some female individuals tended to enter puberty earlier and some to end puberty later. This was not reflected by changes in median ages.

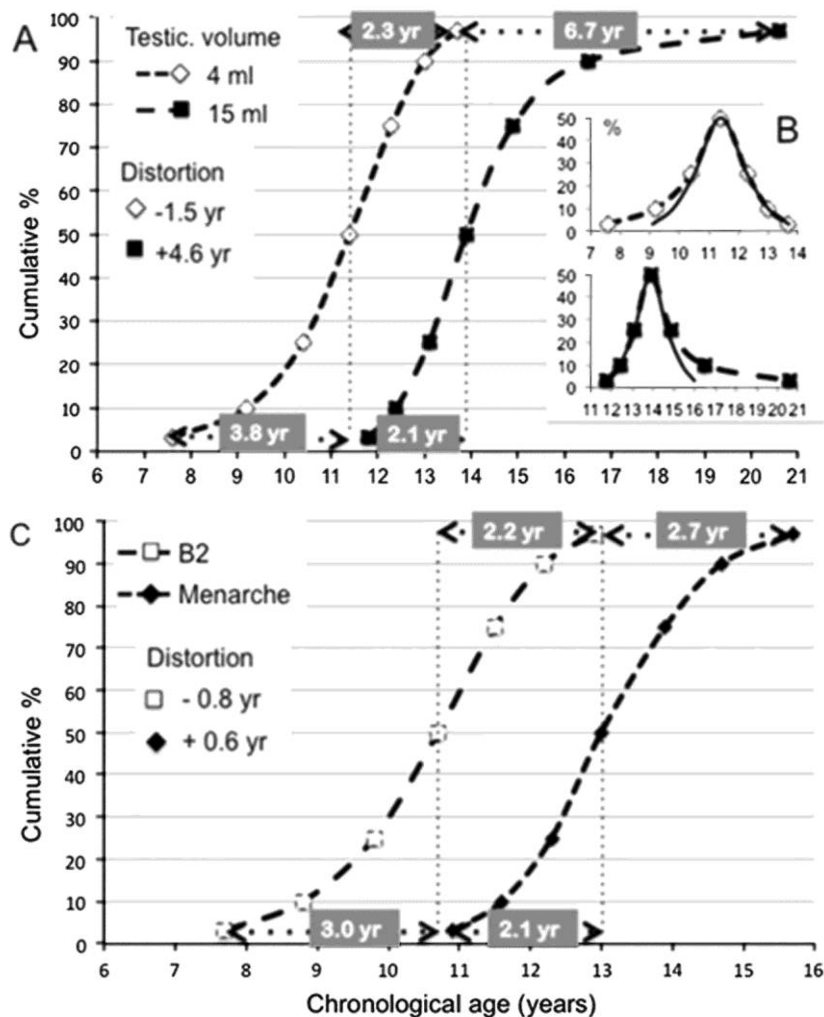
Table 2: Median, upper and lower age limits and distortion of age distribution for initial and final stages of female and male puberty. N: number of subjects, CS: cross-sectional; L: longitudinal; TV: testicular volume.

End-point	Country year of publ.	NCS/L	Year of study	Centile 10 (yr)	Centile 50 (yr)	Centile 90 (yr)	Dist. 10th to 90th (yr)	Distortion (yr)	Ref.
Breast stage ≥B2	Sweden 1996	138 L	1980	9.3	10.8	12.3	3.0	0	Lindgren (1996)
	DK 2009	1100 CS	1992	9.3	10.9	12.4	3.1	-0.1	Aksglaede et al. (2009)
	USA 1997	15439 CS	1992-3	8.5	10.1	11.8	3.3	+0.1	Herman-Giddens et al. (1997)
	Greece 2008	311 L	1995	8.1	9.9	11.3	3.2	-0.4	Papadimitriou et al. (2008)
	NL 2001	3562 CS	1997	9.0	10.7	12.1	3.1	-0.3	Mul et al. (2001)
	Belgium 2009	4471 CS	2004	8.8	10.7	12.2	3.4	-0.4	Roelants et al. (2009)
	DK 2009	995 CS	2007	8.2	9.9	11.6	3.4	0	Aksglaede et al. (2009)
Genital stage 5≈G2	Sweden 1996	116L	1980	9.5	11.6	13.7	4.2	0	Lindgren (1996)
	DK2010	824 CS	1992	10.5	11.9	13.1	2.6	-0.2	Sorensen et al. (2010)
	NL 2001	4019 CS	1997	7.7	11.4	12.8	5.1	-2.3	Mul et al. (2001)
	Belgium 2009	4219 CS	2004	8.9	11.4	13.0	4.1	-0.9	Roelants et al. (2009)
	DK2010	767 CS	2007	10.1	11.6	13.1	3.0	0	Sorensen et al. (2010)
	USA 2012	4131 CS	2005-10	6.6	10.7	13.0	6.4	-1.8	Herman-Giddens et al. (1997)
TV 5=4 ml	DK2010	824 CS	1992	10.8	11.9	13.0	2.2	0	Sorensen et al. (2010)
	NL 2001	4019 CS	1997	8.7	11.4	13.0	4.3	-1.1	Mul et al. (2001)
	Belgium 2009	4219 CS	2004	9.2	11.4	13.0	3.8	-0.6	Roelants et al. (2009)
	China 2011	18807 CS	2003-5	8.8	11.1	12.6	3.8	-0.8	Ma et al. (2011)
	DK2010	767 CS	2007	10.3	11.7	13.0	2.7	-0.1	Sorensen et al. (2010)
Menarche	Belgium 1969	1317 CS	1925-9[a]	12.2	14.5	16.3	4.1	-0.5	Jeurissen (1969)
	Belgium 1969	2254 CS	1945-9[a]	11.7	13.3	14.5	2.8	-0.4	Jeurissen (1969)
	NL2013	1882 CS	1965	11.8	13.4	14.9	3.1	-0.1	Talma et al. (2013)
	Belgium 1985	1048 CS	1960-5[a]	11.6	13.0	14.0	2.4	-0.4	Vercauteren and Susanne (1985)
	NL2013	3029 CS	1980	11.7	13.3	14.9	3.2	0	Talma et al. (2013)
	DK 2009	1100 CS	1992	11.8	13.4	15.1	3.3	+0.1	Aksglaede et al. (2009)
	NL 2001	3562 CS	1997	11.7	13.2	14.9	3.2	+0.2	Mul et al. (2001)
	NL2013	30C8 CS	1997	11.8	13.2	14.9	3.1	+0.3	Talma et al. (2013)

	Belgium 2009	4471 CS	2004	11.6	13.0	14.7	3.1	+0.3	Roelants et al. (2009)
	DK 2009	995 CS	2007	11.8	13.1	15.1	3.3	+0.1	Aksglaede et al. (2009)
	NL2013	2138 CS	2009	11.5	13.0	14.5	3.0	0	Talma et al. (2013)
TV s=12 ml	NL 2001	4019 CS	1997	11.8	13.7	15.4	3.6	-0.2	Mul et al. (2001)
	Belgium 2009	4219 CS	2004	12.1	13.5	15.3	3.2	+0.4	Roelants et al. (2009)
	China 2011	18807 CS	2003-5	11.9	13.7	16.7	4.8	+3.0	Ma et al. (2011)
Genital stage	Sweden 1996	116L	1980	13.6	15.1	16.6	3.0	0	Lindgren (1996)
G5	NL 2001	4019 CS	1997	13.7	15.3	19.5	5.8	+2.6	Mul et al. (2001)
	Belgium 2009	4219 CS	2004	13.5	15.3	18.7	5.2	+1.6	Roelants et al. (2009)

[a] Year of birth.

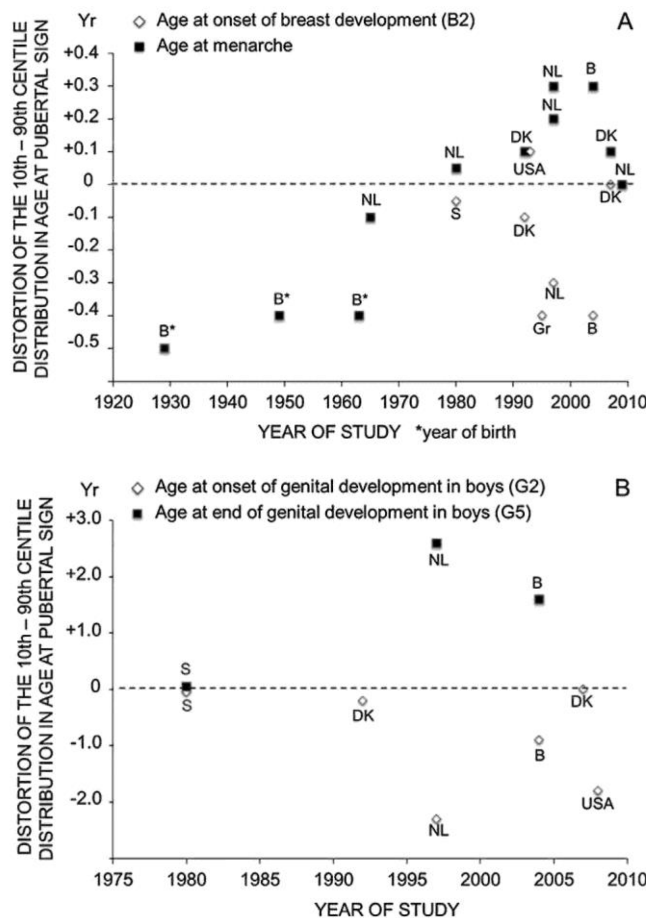
Fig. 3. Cumulative frequency curves (A) and absolute frequency curve (B) of age when achieving a testicular volume ≥4ml (beginning of puberty) or ≥15 ml (late stage of puberty) in Belgium. (C) Cumulative frequency curves of age at breast development (B2) and menarche in Belgium. In boys, the cumulative frequency curves for age at testicular volume ≥4 ml and ≥15 ml (A) seems to be parallel in the rapidly ascending phase but are divergent both in the early and late parts. The two absolute frequency curves (B) illustrate those divergences with opposed changes at the two edges. Calculation of distortion is performed at centile 3 and 97 age points through the difference between the age distances (centile 3-50) and (centile 97-50). Distortion is negative for 4 ml testicular volume and positive for 15 ml. In girls (C), the curve of cumulative frequency of occurrence of breast (B2) seems to parallel that for menarche but the two curves show also divergent patterns at the two edges. Calculation of distortion at centile 3 and 97 reveals a negative value for B2 and a positive value for menarche.



In boys, the median age at onset of genital development (G2 stage) or early pubertal increase in testicular volume (≥4ml) is similar among the different publications except G2 stage in USA (Herman-Giddens et al., 2012). As opposed to the relatively homogeneous variance of onset of puberty in girls, the variance in males is extremely different among the studies: the distance between the 10th and the 90th varies between 2.2 yrs in Denmark (Sorensen et al., 2010) and 6.4 yrs in USA (Herman-Giddens et al., 2012). Distortion of variance is negative in most studies and can attain as much as - 2.3 yrs (Mul et al., 2001). Though there are few studies available, a secular trend towards negative distortion of variance in age at onset of male puberty is suggested (Fig. 4B). While the median age at final pubertal milestones (testicular volume of 12 ml or G5 stage) was very similar among the studies, the variance i.e. the distance between the 10th and the 90th was highly variable among the studies (3.0-5.8 yrs) and distortion was positive and elevated in several studies (Roelants et al., 2009; Mul et al., 2001; Ma et al., 2011). Taken together (Fig. 4B), these data indicate that recent variations in male pubertal timing involve few or no change in median age but a trend towards negative or positive distortion for initial or final pubertal stages, respectively. Such a distorted variance appears to be greater and much more variable among the studies in boys than in girls.

The opposing changes in timing for initial and final endpoints of puberty and the greater variations in boys than in girls lead to revise the concept that current changes involve prominently females and advancement of pubertal timing. Those variations may reflect contemporary effects of environmental factors on pubertal timing.

Fig. 4. The negative or positive distortion of the 10th to 90th centile distribution of age at B2 i.e. onset of breast development and age at menarche (A) or age at occurrence of G2 and G5 stages of genital development in boys (B) are plotted against the year of the study in different countries. A secular trend towards negative distortion appears for the initial pubertal stages (B2 and G2) as opposed to a trend towards positive distortion for the final stages (menarche and B5).



3. Period-dependent impact of environmental factors and mechanisms

In the following sections, we will discuss how the developmental stage can influence the effects of several factors on pubertal timing: energy availability through nutrition, stress, sex steroids, and endocrine disruptors. Though sex steroids do not belong to environmental factors *per se,* they need to be addressed because endocrine disruptors are thought to exert most of their effects through interaction with sex steroid receptors and metabolism.

3.1. Disturbances of energy availability

3.1.1. Critical windows of exposure to abnormal energy availability and timing of puberty in humans

Consistent with Barker's hypothesis of developmental origins of health and diseases (Barker and Osmond, 1986), energy availability in prenatal life can affect age at onset of puberty. Most studies have been carried out in girls and considered age at menarche as the major endpoint. A positive correlation is found between birth weight and age at menarche. Cooper et al. (1996) have shown in a cohort of 1471 girls born in 1946 that the girls who have the lowest birth weight have the earliest age at menarche. Adair has similarly shown a significant positive relationship between birth weight and age at menarche in a longitudinal study on 997 girls born in 1983-1984 in the Philippines (Adair, 2001). Here, a rapid postnatal growth potentiates the effects of birth size. However, birth weight and age at puberty do not always appear to be significantly correlated, possibly because the link is not simply causal and many other factors are confounding. It is possible that common factors determine both low birth weight and early timing of puberty. Some studies do not find any significant correlation between birth length or weight and age at puberty but identify other prenatal or perinatal influencing factors that are related to nutrition: based on pubertal self-assessment in 4000 girls between age 8 and 14, Maisonet et al. find that pre-pregnancy maternal obesity, maternal smoking and primiparity are associated with an earlier age at onset of puberty (Maisonet et al., 2010). It is not possible, however to determine whether each of these prenatal factors has a comparable effect on programming of pubertal timing or whether those factors are closely linked and

trigger each other.

More recently, early postnatal weight gain has appeared to be more strongly linked to onset of puberty in girls than birth weight itself. A prospective study of 2715 girls indicates that low birth weight is associated with menarche before the age of 12 but a positive association between weight gain during the first 9 months of life and age at menarche is also found and statistically much more significant (Ong et al., 2009). Faster weight gain during the first 9 months of life is also positively correlated with fat mass index, body weight and body mass index at puberty (Ong et al., 2009). Other population cohort studies have similarly shown that rapid early postnatal weight gain predicts an early onset of puberty (Dunger et al., 2006; dos Santos Silva et al., 2002). Those results suggest that in addition to prenatal life, early postnatal life could be another window during which variations in nutrition availability program subsequent growth and development. Early nutrition within that window might be an important determinant of both age at puberty and risk of childhood obesity.

Childhood BMI is significantly associated with onset of puberty since heavier children reach puberty earlier (Kaplowitz et al., 2001 ; Rosenfield et al., 2009). In fact, these observations have been used to substantiate the advance in age at breast development that has been reported in the USA during the 1990s (Herman-Giddens et al., 1997). Most of the reports about an association between peripu-bertal weight and puberty onset are cross-sectional and carried out in girls with age at menarche as a marker of puberty. These methodological aspects make a formal association between body weight and puberty difficult to ascertain. Some longitudinal studies, however, have reported a similar association. Aksglaede et al. have found that BMI at 7 years is a significant predictor of age at pubertal growth spurt in a cohort of more than 150,000 boys and girls (Aksglaede et al., 2009). The heaviest prepubertal children enter puberty earlier than the lightest group. Secular trend towards early onset of puberty is found in all BMI categories suggesting that the obesity epidemic is not solely responsible. Another longitudinal study in Sweden show that BMI increase between age 2 and 8 years (adiposity rebound) is positively associated with earlier growth spurt in boys and girls (He and Karlberg, 2001). Recently, Biro confirmed the important impact of BMI on breast development in girls in the USA (Biro et al., 2013). Taken together, those epidemiological studies point towards three windows of sensitivity for a link between energy availability/adiposity and pubertal development: prenatal life, early postnatal life and childhood. Currently, it remains difficult to determine whether those periods represent three independent windows or whether they are part of a continuum. An independent regulation in each of those periods is consistent with opposing effects of prenatal and postnatal weight gain, along with the life history interpretation as discussed above (Sloboda et al., 2011).

The mechanisms involved in the connection between weight gain, adiposity and pubertal timing may be direct and/or indirect. Frish and Revelle have hypothesized that a critical fat mass was necessary for completion of puberty (Frisch and Revelle, 1970). Although challenged later (Ellison, 1982), this hypothesis set the scene for the discovery of peptides responsible for integrating energy availability and the control of reproduction. In the 1990s, leptin has appeared as one of the major factors allowing the reproductive system to sense the magnitude of energy reserves. In humans, leptin is necessary for the onset of puberty but cannot trigger early puberty indicating a permissive role for this hormone (Sanchez-Garrido and Tena-Sempere, 2013). As discussed above, children with intrauterine growth retardation have been shown to be predisposed to subsequent catch up in weight, childhood obesity and early puberty. Several mechanisms have been proposed to explain the association between rapid weight gain and early puberty. Rapid infancy/childhood weight gain has been associated with increased levels of adrenal androgens at age 8 (Ong et al., 2004). Such premature adrenarche could precipitate pitui-tary-gonadal maturation though both processes are found to be dissociated in several conditions, suggesting that physiological levels of adrenal androgens do not influence neuroendocrine maturation (Ducharme and Collu, 1982). In disorders with excess in adrenal androgens like some forms of congenital adrenal hyperplasia, occurrence of secondary central puberty indicates possibly advanced neuroendocrine maturation due to abnormally high levels of sex steroids (Dacou-Voutetakis and Karidis, 1993). Hyperin-sulinemia has been reported in children with a rapid weight gain which is known to lead to decreased levels of sex hormone binding globulin and increased sex steroids bioavailability (Holly et al., 1989). Increased adiposity could also lead to increased aromatase activity and conversion of androgens to estrogens (de Ridder et al., 1992). Catch-up growth in children with low birth weight is associated with a rise in serum leptin (Jaquet et al., 1999) and IGF-1 (Stevens et al., 2014). Rodent models indicate that neonatal leptin could play a crucial role in the development of some neural connections controlling GnRH secretion (Bouret, 2004) and leptin is known to facilitate GnRH secretion peripubertally (Sanchez-Garrido and Tena-Sempere, 2013). IGF-1 is another factor affected by nutrition and that could be able to promote GnRH secretion (Wolfe et al., 2014). In a recent cohort study, mother's age at menarche predicts faster weight gain during infancy in the offspring (Ong et al., 2007). This suggests that the mechanism linking faster infancy weight gain to earlier menarche could include transgener-ational factors, genetic or epigenetic. Gene-environment interactions are hypothesized to account for the recent changes in distribution of age at menarche and adult obesity. Some studies have provided evidence for genetic influences. For instance, the fat mass and obesity-associated gene (FTO) has been associated with a decrease in

age at menarche and increased BMI (Elks et al., 2010). Recent genome-wide association and candidate sin-gle-nucleotide polymorphism studies have identified several loci for age at menarche that are associated with obesity (Elks et al., 2010; den Hoed et al., 2010; Fernandez-Rhodes et al., 2013). The search for known biological pathways shared by loci influencing both adiposity and menarche is still ongoing. Loci that increase adiposity and decrease age at menarche may have particularly strong effects on adult-onset diseases related to prolonged estradiol and fatness exposure such as breast cancer and cardiovascular diseases.

3.1.2. Abnormal energy availability and timing of puberty: mechanisms of action in rodent models

3.1.2.1. Prenatal food restriction. Several rodent models have been used to study the effects of prenatal or early postnatal food restriction on puberty onset (Tables 3 and 4). Prenatal energy restriction is obtained through either decreased food availability to the pregnant dam or ligation of the uterine artery and postnatal food restriction through increased size of the litters (Tables 3 and 4).

While small birth weight for gestational age is associated with early onset of puberty in humans, prenatal underfeeding usually leads to late onset of puberty in female rodents (Table 4). Maternal food restriction (Leonhardt et al., 2003; Iwasa et al., 2010) or uterine artery ligation (Engelbregt et al., 2000) are associated with late vaginal opening or first estrus. Some authors have reported no effect of maternal food restriction on pubertal development while a single study (Sloboda et al., 2009) found an acceleration of pubertal onset. Such discrepancies could be due to differences in food restriction, birth weight or postnatal weight gain. In males, prenatal underfeeding does not affect age at balano-preputial separation (Table 3).

The hypothalamic control of GnRH secretion could be affected by prenatal food restriction in females. After maternal food restriction in rats (50% of daily food intake), Iwasa et al. have shown that the pups have delayed puberty and lower hypothalamic levels of *Kiss1* mRNA on postnatal day 16 and 20 (Iwasa et al., 2010). Pups exposed to prenatal food restriction also show decreased levels of hypothalamic *Kiss1* mRNA while serum leptin are increased, suggesting a central resistance to leptin (Iwasa et al., 2010). The chronic intracerebral administration of kisspeptin peripubertally in pups malnourished prenatally restores normal onset of puberty (Iwasa et al., 2010), suggesting that prenatal food restriction could reversibly affect the kisspeptin system. Whether leptin acts directly on arcuate kisspeptin neurons to mediate regulation of GnRH secretion is still controversial (Donato et al., 2011). We have shown that prenatal food restriction (70% of daily food intake) decreases the stimulatory effect of leptin on GnRH secretion from rat hypothalamic expiants obtained from female pups at 15 days of age (Franssen et al., 2013). Those data indicate that prenatal food restriction could alter the hypothalamic sensitivity to leptin. In our model, we do not observe any significant effect of prenatal undernutrition on leptin receptor mRNA levels. In addition, serum leptin levels at 15 days of age in the animals exposed to prenatal food restriction are not different from controls (Franssen et al., 2013). Thus, the decreased effects of leptin are likely not explained neither by a decreased hypothalamic expression of its receptor nor by variations in serum leptin levels but could be explained by alterations of leptin intracellular signaling pathways.

3.1.2.2. Early postnatal food restriction. Caron et al. and Castellano et al. have shown that postnatal food restriction by increasing the litter size leads to delayed puberty with late vaginal opening and late first estrus cycle in mice (Caron et al., 2012; Castellano et al., 2011). Altered neonatal nutrition leads to alterations in the development of brain circuits responsible for the regulation of puberty and fertility (Castellano et al., 2011). Pups that have been undernourished during early postnatal life display a reduction in the density of kisspeptin fibers compared with normally fed animals. That reduction persists later in life and is associated with decreased fertility (Caron et al., 2012). The most severely affected projections are those originating from the arcuate nucleus and projecting to the median preoptic nucleus (Caron et al., 2012). This indicates that arcuate nucleus circuits are highly plastic and respond to food availability during early postnatal life. The neuronal projections originating from the AVPV and contacting GnRH neural develop before birth (Polston and Simerly, 2006) and are not affected by postnatal undernutrition. Thus, decreased food availability is more likely to cause profound adverse organizational effects when taking place during the development of the nucleus controlling the specific physiological process. Leptin levels are also affected by postnatal nutrition. Bouret et al. have shown that postnatal leptin levels were blunted in the mice from undernourished litters (Bouret et al., 2007). One can speculate that the neural circuits, including the kisspeptin system, that develop during the first three weeks of life could be affected by the reduced levels of leptin neonatally. Further evidence of an early leptin role comes from the capacity of early postnatal leptin treatment to prevent occurrence of obesity in adult rats born with intrauterine growth restriction (Vickers et al., 2005). It is possible that early growth catch up following refeeding provides endogenous hormonal regulators such as leptin and IGF-1 that can influence hypothalamic organization and maturation.

3.1.2.3. Peripubertal food restriction. Food restriction during the peripubertal period leads to a delay of pubertal maturation in males and females (Tables 3 and 4) indicating that critical weight and fat reserves need to be reached in order to complete pubertal development (Frisch and Revelle, 1970). Severe fasting has been shown to

decrease hypothalamic *Kiss1* mRNA expression and kisspeptin immunoreactivity as well as lowering circulating LH in pubertal rats (Castellano et al., 2010, 2005). In pubertal rats, fasting also inhibits the hypothalamic expression of the gene coding for neurokinin B and its receptor (Navarro et al., 2012). Neurokinin B appears to be co-expressed with *Kiss1* in the arcuate nucleus in several species (Lehman et al., 2010) and to be required for normal pubertal maturation (Topaloglu et al., 2010). Those data indicate that neurokinin B and kisspeptin could cooperate in the metabolic control of the neuroendocrine mechanism of puberty. In 2013, Owen et al. have shown that fibroblast growth factor 21 (FGF21), a growth factor secreted by the liver in response to short-term fasting, delayed puberty by suppressing the preovulatory gonadotropin surge (Owen et al., 2013). This effect is exerted through inhibition of vasopressin in the suprachiasmatic nucleus resulting in loss of the stimulatory input to kisspeptin neurons in the AVPV (Owen et al., 2013). Other metabolic hormones have been shown to regulate pubertal activation and to signal energy sufficiency or insufficiency. Among them, ghrelin is an anorectic factor secreted by the stomach. Chronic administration of ghrelin leads to delayed puberty in male and female rats (Tena-Sempere, 2008). It is an inhibitory signal for LH secretion in rodents, sheep and non-human primates (Tena-Sempere, 2008; Iqbal et al., 2006; Vulliémoz et al., 2004). Reduction in the frequency of GnRH pulses has also been reported in immature rats following systemic administration of ghrelin (Lebrethon et al., 2007). This effect can be inhibited by an NPY Y5R antagonist (Lebrethon et al., 2007). This suggests that ghrelin effects on GnRH neuron are mediated, at least in part, by afferent neurons such as NPY neurons. Recent evidence suggests that ghrelin is able to suppress *Kiss1* gene expression at discrete hypothalamic areas (Forbes, 2006). These actions of Ghrelin on the hypothalamic and the pituitary might contribute to the suppression of gonadotropin levels in conditions of persistent negative energy balance, in which ghrelin levels are commonly elevated.

A growing body of evidence has supported the initial hypothesis that insulin is involved in the signaling of satiety to the central nervous system (Niswender et al., 2004). Such physiological effect is consistent with the phenotype of mice harboring a specific deletion of the insulin receptor within neuronal cells (Brüning et al., 2000). These mice have a phenotype very similar to that of mice lacking a functional leptin receptor with hyperphagic obesity and central hypogonadism secondary to functional GnRH deficiency. Using rat primary hypothalamic neurons in cell culture, Burcelin et al. could demonstrate that insulin stimulates *in vitro* both the expression and the secretion of GnRH (Burcelin et al., 2003). Both insulin receptor mRNA and protein are expressed in a clonal cell line expressing GnRH (Salvi et al., 2006). Taken together, these data suggest that peripheral insulin can modulate the activity of hypothalamic GnRH neurons.

Table 3: Age at balano-preputial separation (BPS) after rat exposure to nutritional changes in relation to the age window of exposure.

Ref	Species	Nutrition dams		Nutrition pups		Age at BPS in relation to window of exposure		
		Modalities (%)	Period	Modalities	Period	Prenatal	Postnatal before weaning	Postnatal after weaning
Leonhardt et al. (2003)	Rat	-50	GD 14-21 Lactational			Normal	Late	
Chernoff et al. (2009)	Rat	-50	GD 3-21			Normal		
Sanchez-Garrido et al. (2013)	Rat	-30	GD 1-21	20 vs 12 pups/dam -30%	PND 1-weaning PND 23-...	Normal	Late	Normal
Engelbregt et al. (2004)	Rat			Ligation of uterine art 20 vs 12 pups/dam	GD17 PND 2-24	Late	Late	
Engelbregt et al. (2000)	Rat			20 vs 12 pups/dam	Birth-weaning		Late	
Smith and Spencer (2012)	Rat			20 vs 12 pups/dam	Birth-weaning		Late	
Laws et al. (2007)	Rat			-10%/-40%	PND 23-53			Normal
Smith and Spencer (2012)	Rat			4 vs 12 pups/dam	Birth-weaning		Normal	
Sanchez-Garrido et al. (2013)	Rat			4 vs 12 pups/dam High fat 45% kcal	PND 1-weaning PND 23-...		Early	Normal
Connor et al. (2012)	Rat			High fat 45% kcal	After weaning			Early
Boukouvalas et al. (2008)	Rat			High fat 45% kcal	PND 22-...			Normal

3.1.2.4. Food excess or high fat diet. Studies addressing the impact of early postnatal overfeeding are scarce and report divergent observations depending on the species or strain as well as fat content of the diet. Using small litters (3-5 pups) in order to cause overnutri-tion, some authors did not observe any effect on pubertal onset in females (Gereltsetseg et al., 2012; Sanchez-Garrido et al., 2013) while others have reported earlier puberty (Castellano et al., 2011; Smith and Spencer, 2012). In this case, earlier puberty is accompanied by an increase in serum leptin levels as well as increased expression of k*iss1* mRNA expression in the whole hypothalamus. These data indicate that persistent energy excess during early postnatal development might contribute to a precocious activation of the hypothalamic control of puberty. Independently of the calorie amount, quality of the diet might affect the onset of puberty (Boukouvalas et al., 2008; Fungfuang et al., 2013; Li et al., 2012). High fat diet during gestation or after weaning induces early onset of puberty in females though weight is not significantly different from the control group. There seems to be a strong sexual dimorphism since the timing of male puberty does not seem to be affected by a high fat diet following weaning (Sanchez-Garrido et al., 2013). In females, high fat diet induces advanced maturation of pulsatile LH secretion associated with an accelerated maturation of *Kiss1* and *neurokinin B* mRNA expression in the arcuate nucleus (Li et al., 2012).

3.2. Stress

3.2.1. Critical windows of exposure to stress and timing of puberty in human

It is well established that stress factors have an inhibitory impact on the neuroendocrine regulation of reproduction in women (Warren and Fried, 2001) as in adult nonhuman female primates (Bethea et al., 2008). Strenuous physical exercise, emotional stress or weight loss are associated with hypothalamic amenorrhea (Warren and Fried, 2001 ; Bethea et al., 2008). The impact of stress on timing of puberty is particularly complex to study. Few studies have been able to isolate specific factors involving disturbances of food intake, energy expenditure, economical circumstances or such as chronic disease or psychological stresses. Moreover, many of those studies bear on a limited sample size without control population. Once again, puberty has mostly been studied retrospectively in female subjects with menarche being the main endpoint since it appears to be the most reliable event to be investigated using questionnaires. The effects may vary depending on the nature of the stressors as well as the timing of exposure. Stress in early childhood seems to be associated with earlier menarche (Moffitt et al., 1992; Wierson et al., 1993) whereas exposure shortly before or during puberty has been associated with later onset of menarche (van Noord and Kaaks, 1991; Tahirovic, 1998). Puberty has been found to be delayed during the 1944-1945 Dutch famine study (van Noord and Kaaks, 1991) and the Balkan War (Tahirovic, 1998). In these conditions, the impact of psychological stress during war is confounded by nutritional factors associated with famine.

Children with chronic disease are known to enter puberty later. The prevalence of chronic conditions among adolescents is difficult to assess but is estimated to be between 10% and 15% (Suris et al., 2004). Delayed puberty is common to most chronic illnesses but more frequent in those characterized by malnutrition and chronic inflammation such as bowel disorders or cystic fibrosis. Possible mechanisms will be discussed below based on animal studies. Suppression of the HPG axis and delayed puberty caused by activation of the HPA axis has also been demonstrated in highly trained runners and gymnasts (Klentrou and Plyley, 2003). The impact of physical training on puberty depends on a variety of factors including the type of exercise, the time of onset and the intensity of training. Still, here as for the other conditions discussed under the stress "umbrella", it is obvious that several factors related to nutrition, emotion and activity are intricate and make particularly complex the elucidation of the pathophysiological mechanisms.

Adversity experiences earlier in life, during childhood, have been shown to affect pubertal timing. Most of those studies point to an earlier onset of puberty following psychological stress during childhood. Longitudinal investigations suggest that factors indexing problematic early environments such as marital conflicts, father absence, negative parenting practices or lower socioeconomic status are associated with a younger age at menarche (Moffitt et al., 1992; Wierson et al., 1993). As already discussed for nutritional issues, life history models have proposed that the period between birth and 7 years of age is a period of increased sensitivity to environmental cues. A disturbed environment could result in accelerated reproductive development via signals that resources are limited or uncertain, thus threatening species due to likely reduced reproductive lifespan (Ellis, 2004).

3.2.2. Stress and timing of puberty: mechanisms of action

The mechanisms involved in the effects of stress on puberty might vary depending on the time of occurrence. We will focus initially on the effect of stress taking place during puberty and then consider the mechanisms involved in alteration of puberty caused by prenatal or neonatal stress. There is convincing evidence that corticotrophin-releasing factor (CRF) plays a crucial role in the stress-induced inhibition of GnRH secretion. CRF reduces LH serum concentrations in women during the late follicular and midluteal phases of the cycle

(Barbarino et al., 1989). LH release has been shown to be inhibited by CRF in rodents and monkeys as well. Moreover, the suppression of LH secretion caused by various stresses is prevented by administration of a CRH antagonist (Li et al., 2005; Cates et al., 2004). The timing of puberty is affected by CRF since intracerebroventricular injection of CRF during 14 days, starting on postnatal day 28, delays puberty in female rats while its antagonist causes early puberty (Kinsey-Jones et al., 2010). However, the precise neuroanatomical regions of CRF signaling to influence GnRH secretion are still to be established. The role of the paraventricular nucleus (PVN) is controversial. Although PVN *CRF* mRNA increases in response to stress, it appears that electrolytic lesions of the PVN fail to prevent the inhibition of LH caused by various stresses. The preoptic area (POA) is characterized by synaptic connections between CRF and GnRH neurons (MacLusky et al., 1988) and GnRH neurons express CRF receptors (Jasoni et al., 2005). This suggests an anatomical substrate for a direct functional interaction between the GnRH and the CRF system. Moreover, injection of CRF in the POA inhibits LH release (Rivest et al., 1993). Recently, several stressors such as restraint, hypoglycaemia and lipopolysaccharides have been shown to downregulate *kiss1* and *GPR54* mRNA expression in the medial preoptic area and the arcuate nucleus in adult female rats (Kinsey-Jones et al., 2009). The involvement of kisspeptin in the mediation of the stress effect on puberty is still to be determined.

Glucocorticoids have also been shown to increase the inhibitory action of the RFamide-related peptide (RFRP) on GnRH secretion (Kirby et al., 2009). RFRP has been described as an inhibitor of GnRH secretion in quails, rodents and monkeys (Tsutsui et al., 2000; Clarke et al., 2009; Ubuka et al., 2009). The recent study by Kirby et al. (2009) highlights a possible inhibition of GnRH secretion by peripheral corticoids after acute and chronic stress. This effect is supported by the presence of glucocorticoid receptors in 53% of the cells expressing RFRP (Kirby et al., 2009).

Except for prenatal underfeeding as discussed above, very few studies have focused on the effect of prenatal stress on pubertal development in human. Anoxia is among the most frequent causes of stress at birth that may turn into cerebral palsy. In a cross-sectional study, this condition has been found to involve both early onset and late completion of puberty (Worley et al., 2002). However, there are numerous confounding factors such as reduced activity and feeding problems in addition to the functional impairment of brain areas that may vary among the patients. Rodent data indicate that fetal exposure to glucocorticoids may be an important determinant of sexual maturation. In the offspring of mothers subjected to stress (Politch and Herrenkohl, 1984) or exposed to ACTH or corticoids (Smith and Waddell, 2000) neonatally or during pregnancy, late puberty is observed. Though fetal exposure to maternal corticoids is limited by placental metabolism (Burton and Waddell, 1999), high maternal glucocorticoids concentrations associated with stress are expected to reach the fetus. The mechanisms through which perinatal stress affects pubertal development are still to be elucidated. Prenatal maternal stress increases offspring HPA axis sensitivity, anxiety and cognitive deficits, suggesting alteration of neurodevelopment (Howerton and Bale, 2012). The mechanisms by which early life experience, especially stress, is able to program brain development seem to involve epigenetic modulation of individual genes or large gene clusters. As an example, adult male mice exposed to early prenatal stress show altered expression and DNA methylation in CRF, glucocorticoid receptors (Mueller and Bale, 2008; Nemeroff, 1992) or BDNF (Boersma et al., 2013). Since recent studies have shown that methylation plays a pivotal role in the onset of puberty in female rodent (Ojeda and Lomniczi, 2014), one can hypothesize that early exposure to stress could alter methylation-mediated programming of puberty.

Table 4: Age at vaginal opening (VO) after rat exposure to nutritional changes in relation to the age window of exposure.

Ref	Species	Nutrition dams		Nutrition pups		Age at VO in relation to window of exposure		
		Modalities	Period	Modalities	Period	Prenatal	Postnatal before weaning	Postnatal after weaning
Leonhardt et al. (2003)	Rat	-50%	GD 14-21 AU lactation			Normal	Late	
Gereltsetseg et al. (2012)	Rat	-50%	GD 15-PND 0			Late		
Iwasa et al. (2010)	Rat	-50%	GD 14-PND 0			Late		
Chernoff et al. (2009)	Rat	-50%	GD 3-21	16 vs 8 pups/dam	Lactation	Normal	Normal	
Sanchez-Garrido et al. (2013)	Rat	-30%	GD 1-21	Normal 20 vs 12 pups/dam -30%	PND 1-weaning PND 23-...	Normal	Late	Late
Engelbregt et al. (2004)	Rat			Ligation uter. art. 20 vs 12 pups/dam	GD17 PND 2-24	Late	Normal	
Sloboda et al. (2009)	Rat	Calorie restrict.	Gestation Lactation			Early	Early	
Guzman et al. (2006)	Rat	Protein restrict.	Gestation Lactation			Normal	Late	
Caron et al. (2012)	Mouse			15 vs 8 pups/dam	PND 4-21		Late	
Castellano et al. (2011)	Rat			20 vs 12 pups/dam	PND 1-weaning		Late	
da Silva Faria et al. (2004)	Rat	Protein restrict. Calorie restrict.	Lactation Lactation				Late Late	
Smith and Spencer (2012)	Rat			20 vs 12 pups/dam	Birth-weaning		Normal	
Laws et al. (2007)	Rat			-10%/-40%	PND22-41			Normal
Lo et al. (2009)	Rat	High fat 40% kcal	Gestation	High fat 45% kcal	PND 30-50	Early		Early
Lie et al. (2013)	Rat			High fat 34.9% food	PND1-16 PND1-34 PND 21-34		Normal Normal	Normal
Gereltsetseg et al. (2012)	Rat			5 vs 12 pups/dam	Lactation		Normal	
Caron et al. (2012)	Mouse			3 vs 8 pups/dam	PND 4-21		Normal	
Castellano et al. (2011)	Rat			4 vs 12 pups/dam	PND1-21		Early	
Smith and Spencer (2012)	Rat			4 vs 12 pups/dam	Birth-weaning		Early	
Sanchez-Garrido et al. (2013)	Rat			4 vs 12 pups/dam High fat 45% kcal	PND 1-weaning PND 23-..		Normal	Early
Sloboda et al. (2009)	Rat			High fat diet	After weaning			Early
Boukouvalas et al. (2008)	Rat			High fat 45% kcal	PND 22-..			Early
Fungfuang et al. (2013)	Rat			High fat 60% kcal	PND 21-..			Early
Li et al. (2012)	Rat			High fat 45% kcal	PND 21-..			Early
Connor et al. (2012)	Rat			High fat 45% kcal	After weaning			Early

3.3. Endocrine disrupters

3.3.1. Critical windows for the effects of sex steroids on puberty

Though sex steroids do not belong to environmental factors *per se,* their action needs to be addressed because endocrine disruptors are thought to exert most among their effects through interaction with sex steroid receptors, metabolism and effects. In primate and ovine species, pubertal timing is sexually dimorphic, enabling to study the effects of sex steroids on gender differences in pubertal timing. When female monkey fetuses have been exposed to high androgen levels, menarche is delayed by 4-6 months (Goy et al., 1988). Likewise, fetal lamb exposure to testosterone shifts pubertal timing from a female to a male pattern (Kosut et al., 1997). More insight into the developmental differences in the role of androgens comes from the use of flutamide, an androgen receptor blocker. Prenatal flutamide treatment during early gestation accelerates pubertal timing in male monkeys (Herman et al., 2006). Indirect evidence that this could involve hypothalamic effects arises from the stimulation of LH levels and pulse frequency after flutamide treatment for 3 days in adult men (Urban et al., 1988). Finally, the naturally occurring condition of androgen insensitivity due to androgen receptor mutation in XY humans is associated with a female pattern of peak height velocity at puberty (Zachmann et al., 1986). Taken together, those data are consistent with a prenatal role of androgens in setting pubertal timing.

The hypothalamic targets of perinatal steroids are still incompletely known. Recent rodent studies have underlined the role of perinatal sex steroids in programming the sexual differentiation of Kisspeptin expression in the AVPV (reviewed in Poling and Kauffman (2013)). At later developmental stages, such as puberty, AVPV Kisspeptin neurons may also be further regulated by E2 to exhibit a typical female pattern. The ARC kisspeptin population is not markedly sexually dimorphic in adulthood but this population displays sexually dimorphic characteristics during the neonatal and juvenile periods (reviewed in Poling and Kauffman (2013)). The sex difference in neonatal ARC Kiss1 levels could be due to activational effects caused by temporary variations in circulating sex steroids (reviewed in Poling and Kauffman (2013)).

The window of exposure during development can matter for the effects of sex steroids. While testosterone could reduce LH pulse frequency in both orchidectomized and ovariectomized adult monkeys (Plant, 1986), such a treatment does not affect LH in orchidectomized fetal monkey but reduces LH in the ovariectomized fetal monkey (Ellinwood et al., 1982). By contrast to the delay in pubertal timing caused by androgens prenatally, neonatal treatment of orchidectomized monkeys with testosterone for a year suppresses LH and withdrawal of treatment is followed by an early pubertal resurgence of LH secretion in 2/3 animals (Fraser et al., 2005). This observation is consistent with that of delayed breast development in a girl with a virilizing adrenal tumor who showed very rapid breast development and menarche within 6 months after surgical removal of the tumor (Bourguignon et al., 2015).

The most prevalent human model of fetal or fetal and postnatal androgen exposure is congenital adrenal hyperplasia. This is a complex model however since, after diagnosis and initiation of treatment, further exposure to possible androgen excess as well as to glucocorticoid excess depends on the management. A less heterogeneous condition could be the premature increase in adrenal androgens or premature adrenarche. In this condition where the exposure to unusually high levels of androgens is presumably in the prepubertal period, early menarche has been reported as a function of the severity of intrauterine growth retardation (Ibanez et al., 2006). Treatment with metformin prevents the advancement of menarche (Ibanez et al., 2011) indicating that the determinant of pubertal timing in that condition may be related to prenatal nutritional status (see Section 3.1.2.1) and postnatal insulin sensitivity with no or minor role of adrenal androgens. In summary, the sensitivity to organizing effects of testosterone (directly or after aromatization) centrally is maximal prenatally and neonatally. The central sensitivity to negative feedback effects which is greater in the male than in the female, already operates perinatally and is maximal subsequently, before puberty.

Fig. 5. (A) Etiological distribution of central precocious puberty in 145 Belgian patients. (B) Serum levels of *p,p*-DDE, a derivative of DDT in different patients with sexual precocity. The foreign migrating patients with sexual precocity are adopted or non-adopted. Serum levels of *p,p*-DDE in migrating patients are represented in relation to age at immigration and time since immigration. In 26 foreign patients with precocious puberty, the mean serum concentration of *p,p*-DDE was 10 times higher than the limit of detection, whereas the levels were below this limit in 13 among 15 Belgian native patients. Adapted from data published in Krstevska-Konstantinova et al. (2001).

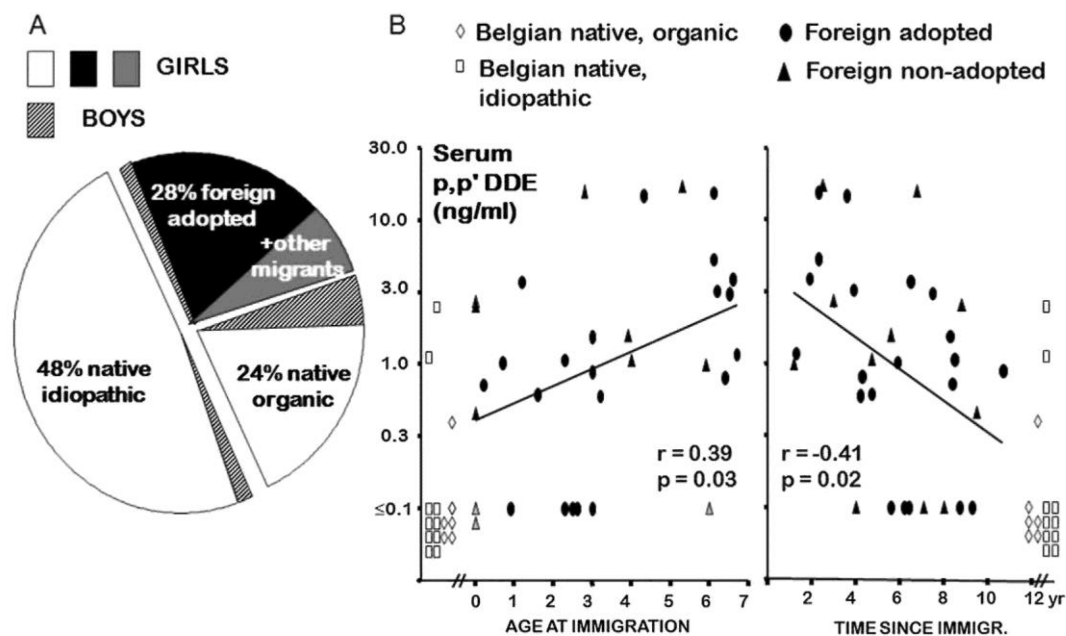


Table 5: Effects of prenatal/early postnatal versus prepubertal exposure to EDCs on timing of breast development in girls.

EDCs	Timing of breast development	Period of exposure	
		Prenatal/early postnatal	Prepubertal
DDE (+DDT)	Early Normal Late	Krstevska-Konstantinova et al. (2001)	Wolff et al. (2008)
Dioxins	Early Normal Late	Leijs et al. (2008)	Den Hond et al. (2002)

Table 6 : Effects of prenatal/early postnatal versus prepubertal exposure to EDCs on timing of menarche in girls.

EDCs	Timing of menarche	Period of exposure	
		Prenatal/early postnatal	Prepubertal
DDE (+DDT)	Early Normal Late	Vasiliu et al. (2004)	Ouyang et al. (2005) Denham et al. (2005)and Ozen et al. (2012)
PCBs	Early Normal Late	Vasiliu et al. (2004) and Yang et al. (2005)	Denham et al. (2005)
Dioxins	Early Normal Late	Leijs et al. (2008) and Warner et al. (2004)	Den Hond et al. (2002)
Phytoestrogens	Early Normal Late	Adgent et al. (2012) Strom et al. (2001) and Giampietro et al. (2004)	Kim et al. (2011)

Table 7 : Effects of prenatal/early postnatal versus prepubertal exposure to EDCs on pubertal timing in boys.

EDCs	Pubertal timing	Period of exposure	
		Prenatal/early postnatal	Prepubertal
DDE (+DDT)	Early		Den Hond et al. (2011)
	Normal	Gladen et al. (2000)	Grandjean et al. (2012)
	Late	Grandjean et al. (2012)	
PCBs	Early		Den Hond et al. (2011)
	Normal	Mol et al. (2002) and Warner et al. (2004)	Grandjean et al. (2012)
	Late	Guo et al. (2004) and Herman et al. (2006)	Den Hond et al. (2002)
Phthalates	Early		Mouritsen et al. (2013)
	Normal Late	Rais-Bahrami et al. (2004)	Mieritz et al. (2012)

Fig. 6. (A) Evolution of mean GnRH interpuise interval throughout development in rats when studied *ex vivo* using individual female hypothalamic expiants (mean ± SD, n = 5 animals per age group). (B) Relative expression of *Kiss1* mRNA in the hypothalamus of female rats at 15 and 25 days of age. (C) Representative profiles of GnRH secretion from hypothalamic expiants obtained from female rats throughout development. The graphs illustrate the decrease of GnRH interpulse interval between PND 5 and 25, before the onset of puberty. Adapted from Bourguignon and Franchimont (1984), except Kiss1 mRNA data.

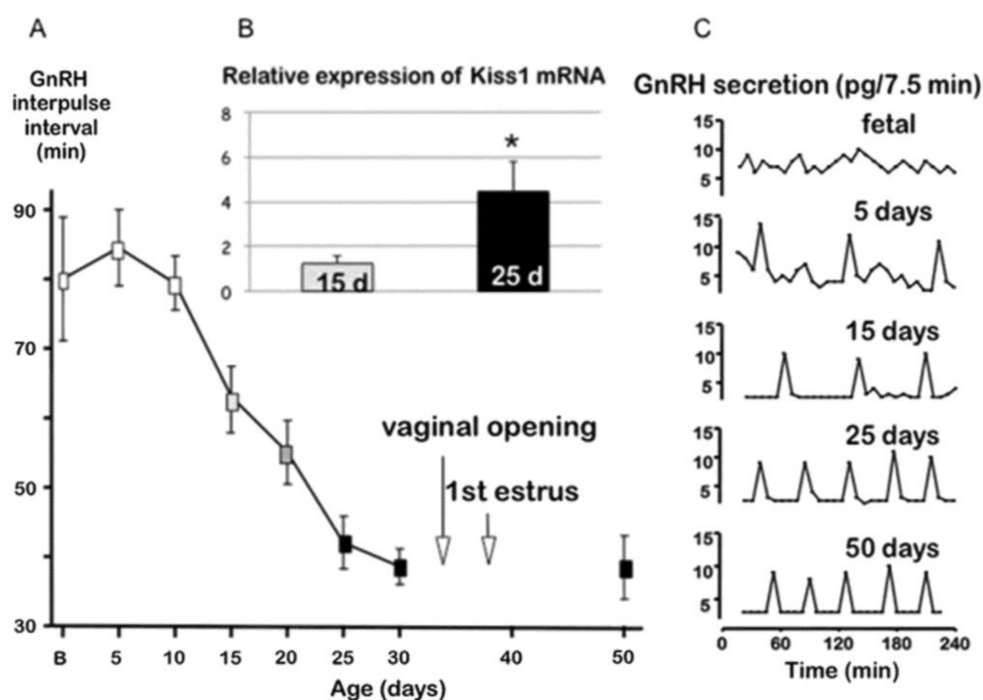


Table 8: Age at balano-preputial separation (BPS) after rat exposure to EDC in relation to the age window of exposure.

EDC	Ref	Exposure route	Dose (µg/kg/d)	Exposure period	Age at BPS in relation to window of exposure		
					Prenatal	Postnatal before weaning	Postnatal after weaning
BPA	Tinwell et al. (2002)	Oral (gav.)	20-50,000	GD 6-21	Normal		
	Nagao et al. (1999)	sc	300,000	PND 1-5		Normal	
	Kato et al. (2006)	sc	0.024-1000	PND 0-9		Normal	
	Tan et al. (2003)	Oral (gav.)	100,000	PND 23-52/53			Normal
	Ashby and Lefevre (2000)	Oral (gav.)	100,000 150,000	PND 35-55			Normal Normal
DDE	Loeffler and Peterson (1999)	Oral (gav.)	1000-100,000 200,000	GD 14-18	Normal Late		
	Yoshimura et al. (2005)	Oral (gav.)	10,000-100,000 10,000-100,000 10,000-300,000 10,000-100,000 300,000 10,000-30,000 100,000	GD 14-17 GD 18-21 PND 1-5 PND 17-21 PND 35-39	Normal Normal	Normal Normal Late	Normal Late
	Ashby and Lefevre (2000)	Oral (gav.)	100,000	PND 22-35 PND 22-55 PND 35-55			Normal Late Normal
Vincloz olin	Yoshimura et al. (2005)	Oral (gav.)	10,000-100,000 10,000-100,000 10,000-100,000 10,000-30,000 100,000 10,000 30,000-100,000	GD 14-17 GD 18-21 PND 1-5 PND 17-21 PND 35-39	Normal Normal	Normal Normal Late	Normal Late
	Monosson et al. (1999)	Oral (gav.)	10,000 30,000-100,000	PND 22-55			Normal Late
DES	Odum et al. (2002)	Oral (water)	6500	GDO-PNDO PND 0-10 PND 0-21 PND 21-100	Normal	Normal Normal	Late
	Yoshimura et al. (2005)	Oral (gav.)	0.1-300 0.1-300 10 100 300 10-300 10 100 300	GD 14-17 GD 18-21 PND 1-5 PND 17-21 PND 35-39	Normal Normal	Normal Late Late Normal	Normal Late Late
	Ashby and Lefevre (2000)	Oral (gav.)	40	PND 22-35 PND 22-55 PND 35-55			Late Late Normal
	Shin et al. (2009)	Oral (gav.)	10 20-40	PND 33-52			Normal Late
DIBP	Saillenfait et al. (2008)	Oral (gav.)	125,000 250,000 500,000 625,000	GD 12-21	Early Normal Late Late		
DEHP	Ge et al. (2007)	Oral (gav.)	10,000 500,000 750,000	PND 21-49			Early Normal Late

	Noriega et al. (2009)	Oral (gav.)	10,000-100,000 300,000-900,000	PND 21-		Normal Late
	Botelho et al. (2009)	Oral (gav.)	250,000-750,000	PND 21-51		Normal
DBP	Salazar et al. (2004)	Oral (food)	12,000 50,000	Gestation + 2 mths ahead	Normal Late	
	Mylchreest et al. (2000)	Oral (gav.)	500-500,000	GD 12-21	Normal	
	Ashby and Lefevre (2000)	Oral (gav.)	500,000	PND 22-35 PND 22-55 PND 35-55		Normal Normal Normal
PBDE-99	Lilienthal et al. (2006)	sc	1000-10,000	GD 10-18	Normal	
PBDE-71	Stoker et al. (2004)	Oral (gav.)	3000 30,000 60,000	PND 22-53		Normal Late Late

BPA: bisphenol A; DDE: dichlorodiphenyltrichloroethane; DES: diethylstilbestrol; DIBP: diisobutylphthalate; DEHP: Diethylhexylphthalate; PBDE-99: 2,2,4,4,5-pentabro-modiphenylether; PBDE-71: polybrominated Diphenylether; sc: subcutaneous; gav.: gavage.

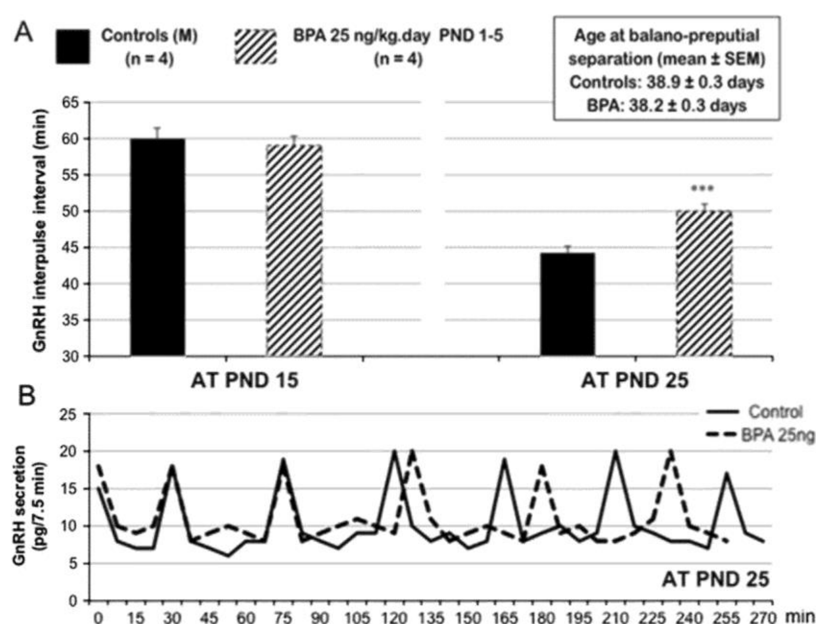
3.3.2. Critical windows of exposure to endocrine disrupters and timing of puberty in humans

Except the condition of industrial spill, it is very difficult to determine in humans whether exposure to EDCs has taken place during prenatal and early postnatal life or later during the prepubertal period. The demonstration of exposure during the prepubertal period does not exclude former exposure in fetal life or infancy. Additional difficulties come from the likely exposure to mixtures of EDCs among which the authors isolate one or few EDCs detected in blood and/or urine. Moreover, the EDCs persisting in the environment and the body tissues (POPs or persistent organic pollutants) are more likely to be detected. So, with caution, we have attempted to delineate (Tables 5-7) whether given EDCs would influence pubertal timing in humans differently depending on the presumable period of exposure. As far as the insecticide DDT and its derivative DDE, the interpretation is complex since the former has prominently estrogenic properties and the latter is considered to be anti-androgenic (Rasier et al., 2008). Moreover, DDE is present as a minor constituent in commercial DDT preparations and arises as well from degradation of DDT in the body. As shown in Fig. 5A, we have reported that about a quarter of the patients treated for sexual precocity in Belgium had migrated from abroad, prominently for international adoption and came from malaria endemic countries (Krstevska-Konstantinova et al., 2001). The risk of precocity has been estimated to be 80 times higher than in Belgian native children. In that condition, depending on age at adoption, most children had been exposed to DDT prominently or exclusively during prenatal life and infancy. Based on DDE serum levels that are positively related to the age at immigration and negatively related to time since immigration (Fig. 5B), we have proposed the involvement of DDT in the early pathogenesis of that increased rate of sexual precocity (Krstevska-Konstantinova et al., 2001). Wolff et al. (2008) do not find any change in timing of breast development in relation to serum DDE levels in girls in New York city (Table 5). Data on the timing of menarche (Table 6) after prepubertal exposure to DDE (DDT) are discrepant since it has been found to be early (Ouyang et al., 2005; Deng et al., 2012) or normal (Denham et al., 2005; Ozen et al., 2012). However, on account of the persistence of the insecticide in the body fluids, exposure earlier than in prepuberty cannot be excluded in these studies. Vasiliu et al. (2004) have reported early timing of menarche after presumable prenatal/early postnatal exposure. In boys, Den Hond et al. (2011) have observed early puberty after prepubertal exposure to DDE (DDT) but normal timing after exposure in early life (Gladen et al., 2000). An interesting study based on PCBs and DDE levels in cord blood versus serum levels in adolescence has shown that the neonatal levels only are predictive of slightly smaller testes (Grandjean et al., 2012). In addition, lower LH and testosterone levels in serum suggest a delayed puberty of central origin (Grandjean et al., 2012). In the other studies on PCBs and dioxins, the vast majority of investigations after prenatal and/or early postnatal exposure conclude to normal pubertal timing both in girls (Vasiliu et al., 2004; Yang et al., 2005; Warner et al., 2004) and in boys (Gladen et al., 2000; Mol et al., 2002) while Guo et al. report delayed puberty in boys (Guo et al., 2004) and Leijs et al. late breast development in girls (Leijs et al., 2008). The data after prepubertal exposure are equivocal. Den Hond et al. observe late breast development but normal timing of menarche (Den Hond et al., 2002). These authors also find puberty to be delayed in boys (Den Hond et al., 2002) but they have reported early male puberty more recently (Den Hond et al., 2011). After exposure to phytoestrogens (Adgent et al., 2012; Kim et al., 2011; Strom et al., 2001; Giampietro et al., 2004) or phthalates (Mouritsen et al., 2013; Rais-Bahrami et al., 2004; Mieritz et al., 2012), the findings are also inconsistent: normal or early timing of menarche and

normal or early male puberty irrespective of the presumable period of exposure.

It is challenging to show the involvement of neuroendocrine mechanisms in disordered pubertal timing after exposure to EDCs for several reasons. EDCs can interfere with any site where hormones are acting including the peripheral target tissues of sex steroids. Thus, disorders of puberty such as occurrence of estrogenic or anti-androgenic effects out of the normal timing limits could result from peripheral effects of EDCs. EDCs can interfere with the physiological (prominently inhibitory) feedback mechanisms of sex steroids on hypothalamic-pituitary function while they can also stimulate neuroendocrine maturation (Rasier et al., 2006). Additional difficulties in human studies come from the limited access to endpoints of neuroendocrine maturation that is commonly assessed indirectly through LH measurement (Grandjean et al., 2012). With the aim of obtaining direct insight into the neuroendocrine changes involved in the mechanism of onset of puberty, we have developed a model enabling to study pulsatile GnRH secretion from hypothalamic explants (Bourguignon and Franchimont, 1984; Bourguignon et al., 1990, 1992). Representative profiles of GnRH secretion are illustrated in Fig. 6 as well as the reduction in GnRH interpulse interval occurring between 10 and 25 days of age, before vaginal opening i.e. the first phenotypic evidence of sexual maturation in the female rat. That acceleration of pulsatile GnRH secretion coincides with an increase in *Kiss-1* mRNA expression in the hypothalamus.

Fig. 7. (A) Average GnRH interpulse interval *in vitro* on postnatal day (PND) 15 and 25 using hypothalamic expiants obtained from male rats after neonatal exposure (PND 1-5) to vehicle or 25 ng/kg/d of BPA injected S.C. ***$p < 0.001$, versus aged-matched control group. Data are mean ± SEM ($n = 4$). (B) Representative profiles of GnRH secretion obtained on PND 25 using hypothalamic expiants of male rats after neonatal exposure (PND 1-5) to vehicle or 25 ng/kg/d of BPA. BPA (25 ng/kg/d) increases GnRH interpulse interval in hypothalamic expiants obtained from male rats exposed neonatally to BPA and studied on PND25, before the onset of puberty.



3.3.3. Endocrine disrupters and timing of puberty: mechanisms of action in rodent models

3.3.3.1. Male rodent. As shown in Table 8, with the exception of early balano-preputial separation after exposure to phthalates (Ge et al., 2007; Saillenfait et al., 2008), EDCs appear to cause either no effect or a delay of sexual maturation in the male rodent. BPA does not show any effect whatever the dose and the window of exposure (Tinwell et al., 2002; Nagao et al., 1999; Tan et al., 2003; Ashby and Lefevre, 2000). As shown in Fig. 7, in an experiment where the same environmentally relevant dose of BPA as the lowest dose used by Kato et al. (2006) is used neonatally for 5 days, we come to the same conclusion regarding the phenotypic onset of puberty. However, when pulsatile GnRH secretion from hypothalamic expiants is studied at 25 days in such exposed animals, GnRH pulse frequency is reduced (Fig. 7). Since that increase in frequency is a developmental neuroendocrine event (Bourguignon and Franchimont, 1984), the reduction could result from delayed maturation caused by BPA neonatally. Then, because there are no phenotypic consequences, it is possible that the neuroendocrine system is sensitive enough to explain a delayed hypothalamic maturation that, however, was not

sufficient or not lasting sufficiently to account for phenotypic changes. The very low concentration of BPA dose should be emphasized. Current experiments attempt to delineate whether neonatal BPA exposure for 2 weeks could affect both neuroendocrine function and the phenotype. We also aim at studying the gene response to such a low dose of BPA in comparison with a higher dose.

Fig. 8. Cumulative percentage of female rats displaying vaginal opening in relation to age (PND 31-41) in animals exposed neonatally to vehicle or 1 µg/kg/d of DES (panel A) or 10 µg/kg/d of DES (panel B). The number of animals in each group is indicated. Panel C: GnRH interpulse interval *in vitro* at PND 15 or 25 using hypothalamic expiants obtained from female rats after neonatal exposure (PND 1-5) to vehicle or 1 µg/kg/d or l0µg/kg/d of DES. $^{**}p<0.01$, versus aged-matched control group. Data are mean ± SEM (n = 4). Panel D: Relative expression of *KiSS1* mRNAs in the hypothalamus of female rats on PND 15 after neonatal exposure to DES 1 µg/kg/d or 10 µg/kg/d (B and E). $^{*}p < 0.05$, versus controls. Data are mean ± SEM (n = 5 animals in each group). A dose of 1 µg/kg of DES causes delayed VO, and increased GnRH interpulse interval at 25 days and reduced hypothalamic expression of *Kiss-1* mRNA on PND15. A higher dose of 10 µg/kg of DES results in early VO, does not affect GnRH pulse frequency but reduces *Kiss-1* mRNA expression such as the lower dose. Adapted from data published in Franssen et al. (2013).

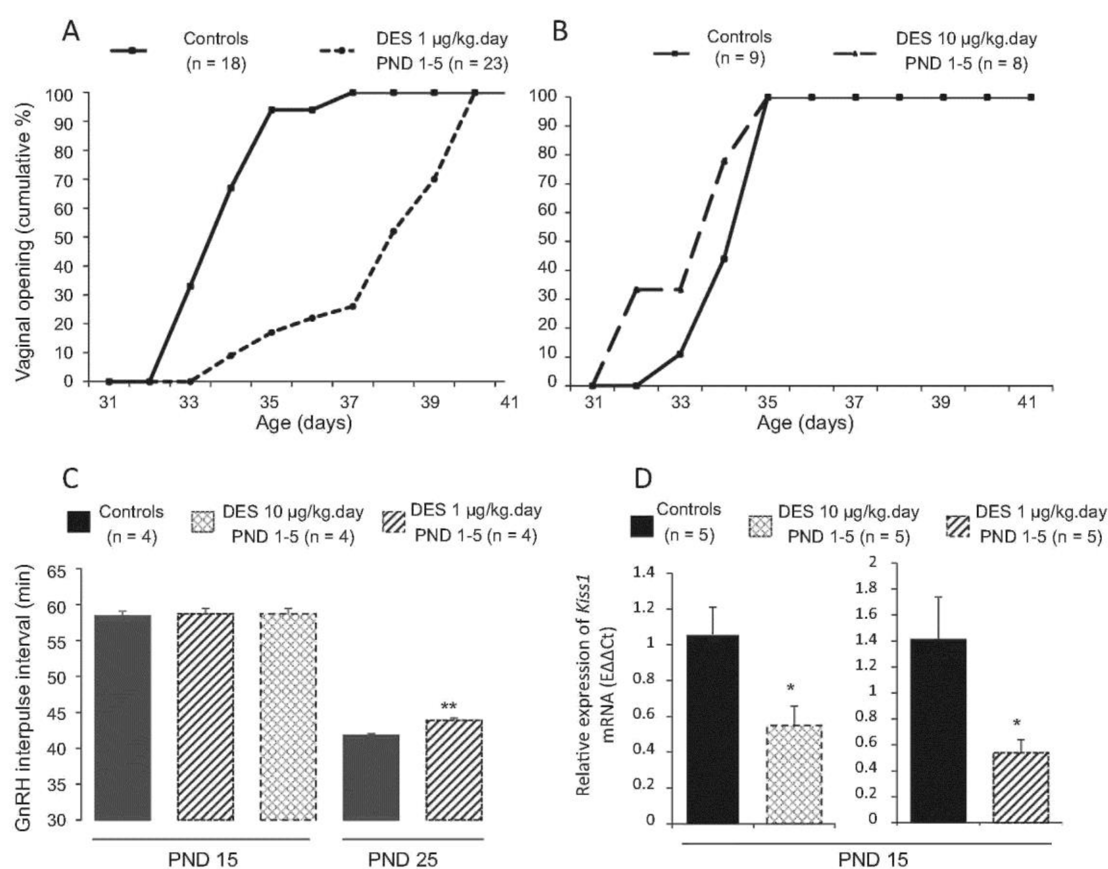


Table 9: Age at vaginal opening (VO) after EDC exposure in relation to the age window of exposure.

EDC	Reference	Species	Exposure route	Dose (µg/kg/d)	Exposure period	Age at VO in relation to window of exposure		
						Prenatal	Postnatal before weaning	Postnatal after weaning
BPA	Tinwell et al. (2002)	Rat	Oral (gav.)	20-50,000	GD 6-21	Normal		
	Murray et al. (2007)	Rat	Implant	2.5-1000	GD 9-PND1	Normal		
	Howdeshell et al. (1999)	Mouse	Oral (food)	2.4	GD 11-17	Normal		
	Honma et al. (2002)	Mouse	sc	2 20	GD 11-17	Normal Early		
	Nikaido et al. (2004)	Mouse	sc	500	GD 15-18	Early		
	Adewale et al. (2009)	Rat	sc	50 50,000	PND 0-3		Early Normal	
	Losa-Ward et al. (2012)	Rat	sc	50 50,000	PND 0-3		Early Normal	
	Nagao et al. (2001)	Rat	sc	300,000	PND1-5		Normal	
	Fernandez et al. (2009)	Rat	sc	~5000/50,000	PND 1-10		Early	
	Yu et al. (2010)	Rat	Oral (gav.)	150,000	PND 5-11		Normal	
	Nah et al. (2011)	Mouse	sc	100-100,000	PND 8		Early	
	Nikaido et al. (2005)	Mouse	sc	10,000	PND 15-18		Normal	
	Laws et al. (2000)	Rat	Oral (gav.)	200,000-400,000	PND 21-35			Normal
DES	Odum et al. (2002)	Rat	Oral (water)	6500	GD 0-PND 0 PND 0-10 PND 0-21 PND 21-100	Normal	Normal Early	Early
	Maranghi et al. (2008)	Mouse	Oral (gav.)	10	GD 9-16	Early		
	Honma et al. (2002)	Mouse	sc	0.02-2	GD 11-17	Early		
	Nikaido et al. (2004)	Mouse	sc	0.5	GD 15-18	Early		

	Yamamoto et al. (2003)	Rat	sc	1.5	GD 7-21	Normal		
	Rothschild et al. (1988)	Rat	sc	4.8	GD 15 + GD 18	Early		
	Burroughs et al. (1985)	Mouse	sc	0.08	PND 1-5		Early	
	Franssen et al. (2013)	Rat	sc	1 10	PND 1-5		Late Early	
	Willoughby et al. (2005)	Rat	sc	500	PND 1-10		Early	
	Kim et al. (2002)	Rat	Oral (gav.)	0.2 1-5	PND 21-40			Normal Early
Geni-stein	Nikaido et al. (2004)	Mouse	sc	500	GD 15-GD 18	Early		
	Losa et al. (2011)	Rat	sc	1000-10,000	PND 0-3		Early	
	Bateman and Patisaul (2008)	Rat	sc	10,000	PND 0-3		Early	
	Nagao et al. (2001)	Rat	Oral (gav.)	12,500-100,000	PND 1-5		Normal	
	Jefferson et al. (2005)	Mouse	sc	500-50,000	PND 1-5		Normal	
	Jefferson et al. (2005)	Mouse	Oral (mouth)	6250-25,000 37,500	PND 1-5		Normal Late	
	Kouki et al. (2003)	Rat	sc	1000	PND 1-5		Early	
	Kouki et al. (2005)	Rat	sc	1000	PND 5		Normal	
	Lewis et al. (2003)	Rat	sc Oral	200 2000 4000 40,000	PND 1-6 PND 7-21		Normal Early Normal Early	
DBP	Salazar et al. (2004)	Rat	Oral (food)	12,000 50,000	Gestation (+2mo before)	Late Late		
	Hu et al. (2013)	Rat	sc	500-50,000 500-50,000	PND 1-5 PND 26-30		Early	Early
	Gray et al. (2006)	Rat	Oral (gav.)	500,000	PND22-...			Normal
BBP	Moral et al. (2007)	Rat	Oral (gav.)	2.5	PND 2-20		Normal	
DEHP	Ma et al. (2006)	Rat	Inhalation	5-25 mg/m^3	PND 22-84			Early
PBDE-99	Lilienthal et al. (2006)	Rat	sc	1000 10,000	GD 10-18	Normal Late		
	Ceccatelli et al. (2006)	Rat	sc	1000-10,000	GD 10-18	Normal		

PBDE-71	Stoker et al. (2004)	Rat	Oral (gav.)	3000-30,000 60,000	PND 22-41		Normal Late
DDT	Gellert et al. (1972)	Rat		1000 5000	PND 23-50		Normal Early
	Heinrichs et al. (1971)	Rat		100,000	PND 2-4	Early	
	Gellert et al. (1974)	Rat	Gavage	10,000	PND 2-4	Normal	
	Rasier et al. (2007)	Rat	sc	10,000-100,000	PND 6-10	Early	

BPA: bisphenol A; **DDE:** dichlorodiphenyltrichloroethane; DES: diethylstilbestrol; DBP: dibutyl phthalate; PBDE-99: 2,2,4,4,5-pentabromodiphenylether; **PBDE-71:** poly-brominated diphenylether; SEM: semicarbazide; NP: p-nonylphenol; sc: subcutaneous; gav.: gavage.

When similar doses of different EDCs are used at different periods of exposure in the male rodent, they are found to be effective in delaying puberty after postnatal exposure (post-weaning) as opposed to absence of effects after prenatal exposure. Developmental differences in EDC effects are obtained using DDE (Den Hond et al., 2002), vinclozolin (Den Hond et al., 2002) or diethyl-stilbestrol (DES) Yoshimura et al., 2005; Odum et al., 2002. Such a conclusion is also drawn in a review of several studies on BPA effects in the female rat since gestational exposure has no effect on age at vaginal opening whereas neonatal exposure is followed by early puberty (Bourguignon et al., 2013). Those data are consistent with possible developmental variations in rodent sensitivity to EDCs that cannot be extrapolated to humans. Differences in developmental timing need to be taken into account. Although both fetal and early postnatal periods of exposure fall into the so-called "programming window", there may be differences in sensitivity to endocrine disruption even within this particular period. The dose of EDC plays a critical role since, when investigated at a given period of life, higher doses appear to be more effective such as shown after prenatal exposure for DDE (Loeffler and Peterson, 1999) and phthalates (Saillenfait et al., 2008; Salazar et al., 2004), or after lactational exposure for DDE, vinclozolin or DES (Yoshimura et al., 2005) or following exposure after weaning for DDE (Ashby and Lefevre, 2000; Yoshimura et al., 2005), vinclozolin (Yoshimura et al., 2005; Monosson et al., 1999), DES (Yoshimura et al., 2005; Shin et al., 2009), phthalates (Ge et al., 2007; Noriega et al., 2009) and PBDE (Stoker et al., 2004). It is noteworthy that in two studies using phthalates, opposing effects are observed since lower doses are associated with early puberty and higher doses with delayed puberty (Ge et al., 2007; Saillenfait et al., 2008). We have reported similar dose-dependent opposing effects using DES neo-natally in the female rat (Sorensen et al., 2010).

3.3.3.2. Female rodent. The effects of EDCs on the timing of vaginal opening (VO) in relation to the age window of exposure are summarized in Table 9.

When the animals are exposed to BPA during pregnancy only, age at VO is either not affected (Tinwell et al., 2002; Murray et al., 2007; Howdeshell et al., 1999) or early (Honma et al., 2002; Nikaido et al., 2004), the latter authors restricting treatment to late gestation only. When treatment starts postnatally after 15 days, age at VO is not affected (Nikaido et al., 2005; Laws et al., 2000). During the age window comprised between birth and 15 days, age at VO is either not affected (Nagao et al., 1999; Adewale et al., 2009; Losa-Ward et al., 2012; Yu et al., 2010) or early (Adewale et al., 2009; Losa-Ward et al., 2012; Fernandez et al., 2009; Nah et al., 2011). A dose of 50 µg/kg causes advancement of puberty while 50 mg/kg has no effect, indicating a non-linear dose-response relationship (Adewale et al., 2009; Losa-Ward et al., 2012). Taken as a whole, those studies show that BPA effects depend markedly on the window of exposure and the dose, with possible non-linear dose-response relationship. In addition to our above-mentioned data showing reduced GnRH pulse frequency after neonatal exposure to a low dose of BPA in the male rat (Fig. 7), several studies have provided evidence of neuroendocrine changes after early exposure to BPA in the female rat. Losa-Ward et al. (2012) have reported that exposure to 50 µg/kg BPA results in alteration of RFamide-related peptide-3 (RFRP3) neurons known to inhibit GnRH neuron activity. There are reduced RFRP3 perika-ria, fiber density and contacts on GnRH neurons, suggesting that BPA-induced premature puberty could result from decreased inhibition of GnRH neurons. Fernandez et al. have found that GnRH pulse frequency is accelerated and LH secretion reduced basally and in response to GnRH (Fernandez et al., 2009). As we have discussed in a study using DDT, reduction in LH secretion can be a developmental component of pituitary maturation before puberty and does not necessarily mean negative feedback effects (Rasier et al., 2007).

In the vast majority of studies, prenatal or early postnatal exposure to DES is followed by early VO (Honma et al., 2002; Nikaido et al., 2004; Maranghi et al., 2008; Rothschild et al., 1988; Burroughs et al., 1985; Willoughby et al., 2005). This possibly results from premature hypothalamic-pituitary maturation because DES is not present at the time of VO anymore. When DES is given to female rats after weaning (Odum et al., 2002; Kim et al., 2002), early VO is observed and likely involves a peripheral effect because that potent synthetic estrogen is still given at the time of VO. Our recent study (Franssen et al., 2013) provides direct indication that neonatal DES exposure alters the neuroendocrine system as illustrated in Fig. 8. A dose of 1 µg/kg of DES which surprisingly causes delayed VO consistently accounts for reduced hypothalamic *Kiss-1* mRNA expression at 15 days and increased GnRH interpulse interval at 25 days. A higher dose of 10 µg/kg of DES results in early VO. The latter dose however does not affect GnRH pulse frequency and reduces *Kiss-1* mRNA expression such as the lower dose. The above data suggest that the effects of estrogenic EDCs on pubertal onset are equivocal, depending on doses and time of exposure. Some of the reported effects on GnRH pulsatile secretion and *Kiss1* expression suggest that the effects of EDCs on pubertal onset involve hypothalamic effects.

Using a murine model of early and transient neonatal DDT exposure, we have confirmed early developmental acceleration of pulsatile GnRH secretion followed by early sexual maturation (Rasier et al., 2007). Further studies have indicated that such effects involve the estrogen receptor, the orphan dioxin (AhR, aryhydrocarbon) receptor and a subtype of glutamate receptor (Rasier et al., 2008).

Fig. 9. (A) Average age at vaginal opening (VO) and first estrus in female rats exposed to vehicle or 10 or 100 mg/kg/day of o,p'-DDT from PND 6 to 10. Exposure to 10 mg/kg/d of o,p'-DDT led to a significant advance of vaginal opening and first estrus. Exposure to 100 mg/kg/day led to a significant advance of 1st estrus. (B) Average time interval between vaginal opening and 1st estrus in female rats exposed to vehicle or 10 or 100 mg/kg/day of o,p'-DDT from postnatal day 6 to 10. Exposure to o.p'DDT leads to an increased interval between vaginal opening and 1st estrus. (C) Average serum LH in female on PND 15 after exposure to vehicle or 10 or 100 mg/kg/day of o,p'-DDT from postnatal day 6 to 10. (D) Representative secretory profiles of GnRH from hypothalamic expiants obtained from 15 day-old females and incubated in control conditions (MEM or minimum essential medium) or in the presence of o,p'-DDT or an estrogen receptor antagonist (ICI 182.780, 10^{-7} M) or a Aryl hydrocarbon receptor antagonist (α-naphtoflavone, 10^{-7} M). Average GnRH interpulse interval is indicated for each condition. 0,p'-DDT significantly decreases the GnRH interpulse interval. This effect is bloqued by ICI 182.780 and ot-naphtoflavone, indicating that DDT affects on GnRH secretion involves both the estrogen and aryl hydrocarbon receptors. Data are mean ± SD; *n* = 10 animals per group. *$p < 0.005$. Adapted from data published in Rasier et al. (2007).

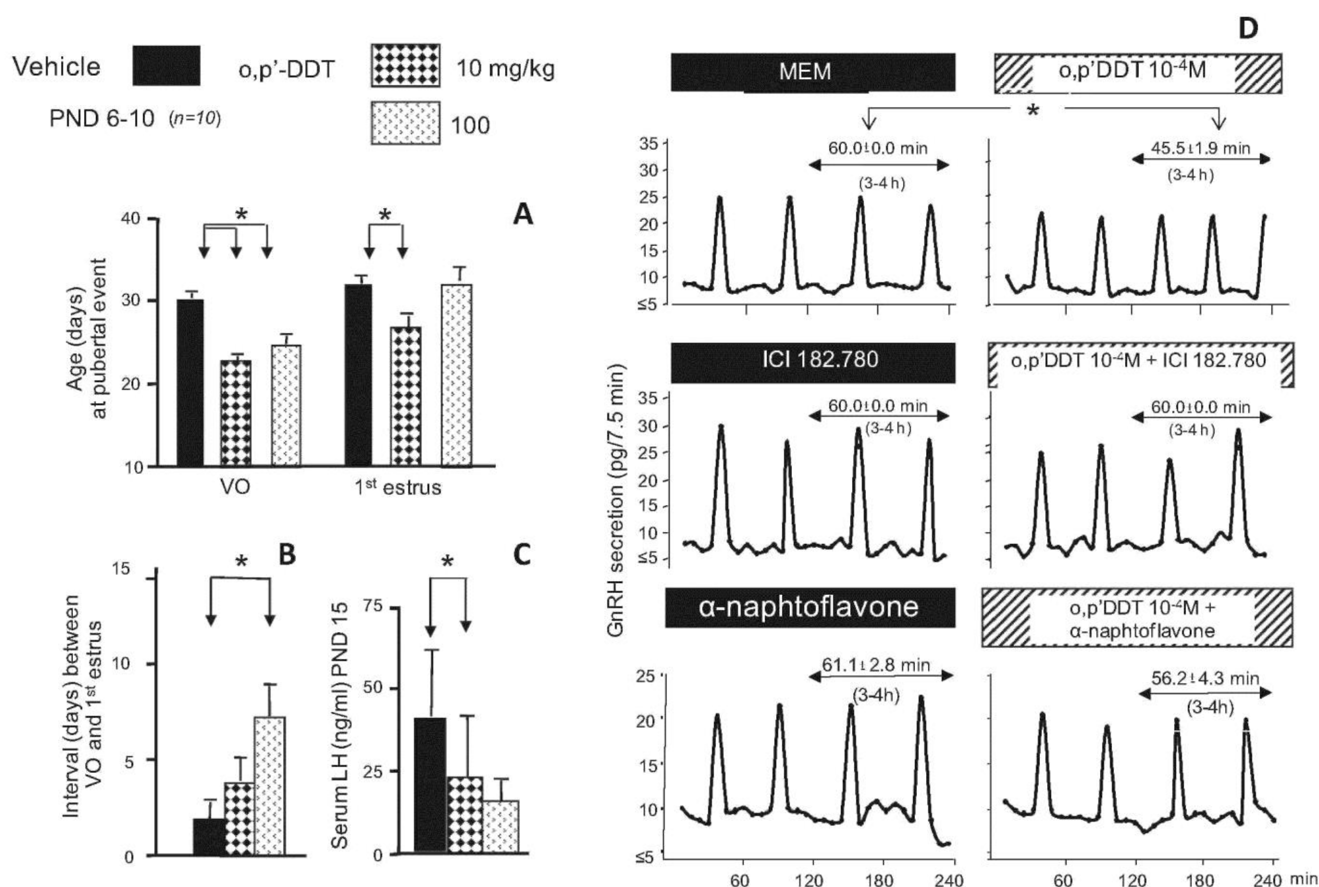
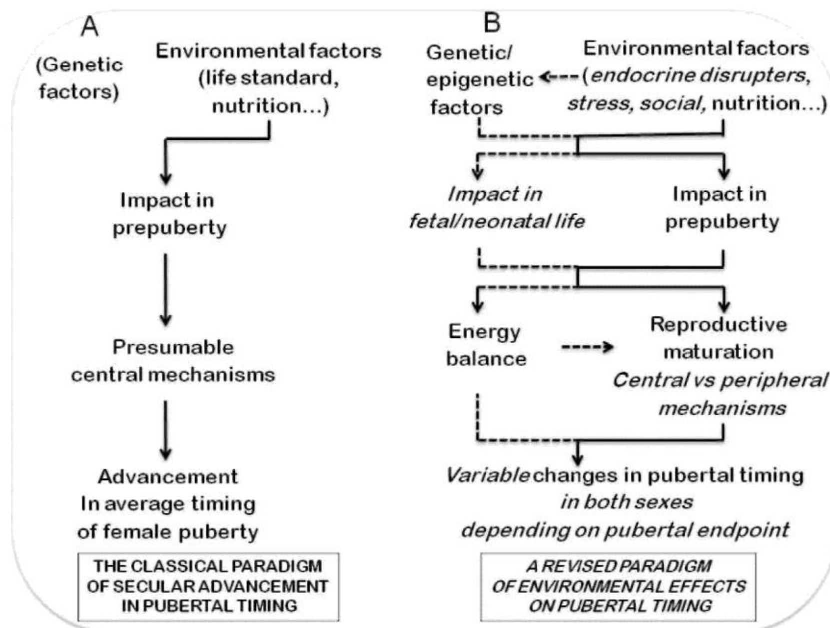


Table 10: Environmental influences on neuroendocrine control of pubertal timing and *mechanisms* in relation to the period of exposure during development: summary.

Factors	Critical periods of exposure	
	Fetal-neonatal	Prepubertal
Sex steroids	Early onset, anovulation Changes in sexually dimorphic characteristics Androgen effect, direct and/or possibly mediated by estrogens after aromatization, epigenetics	Late onset centrally (and early peripheral effects) Negative feedback centrally (and estrogen/androgen balance peripherally)
Insufficient energy availability	Early or late onset Organizing effects of leptin in the neuroendocrine system, epigenetics	Late onset, anovulation Leptin as a prerequisite to normal GnRH secretion
Stress	Early onset? Epigenetics	Late onset centrally Inhibitory CRF effects
Endocrine disruptors	Early or late onset, anovulation Dependency on chemical dose, mixtures; estrogenic effect, epigenetics	Early or late onset centrally (and early or late peripheral effects) Negative feedback centrally (and estrogen/androgen balance peripherally)

Fig. 10. Illustration of the classical and revised interpretations of the current changes in pubertal timing. According to the classical paradigm, improvement in life standards and socio-economical conditions has paralleled a secular advancement in pubertal timing illustrating the fact that environmental factors are predominant in determining this trend in pubertal timing. According to the revised paradigm, environmental factors can influence pubertal timing and reproduction through different mechanisms (central versus peripheral) depending whether they take place early, during fetal and neonatal life or late during prepubertal life. Environmental factors can also affect energy balance during the fetal/neonatal life or during puberty and energy balance can consequently affect reproductive maturation. Environment might affect genetic or epigenetic pathways during these critical windows and thereby influence the pubertal timing, both towards earliness or lateness, depending on the pubertal endpoint studied.



Using the phytoestrogen genistein, most studies have investigated the consequences of neonatal exposure with equivocal effects since age at VO was either normal with no dose-related effect (Nagao et al., 2001; Jefferson et al., 2005; Kouki et al., 2003) or early and dose- or duration-dependent (Kouki et al., 2003; Lewis et al., 2003) or even late and dose-dependent (Jefferson et al., 2009). Still, the evidence of neuroendocrine effects is lying on reduced fibers immunopositive for *Kiss-1* (Losa et al., 2011 ; Bateman and Patisaul, 2008) which is inconsistent with early sexual maturation as opposed to increased *FOS* expression in GnRH neurons (Losa et al., 2011; Bateman and Patisaul, 2008). Using phthalates, the period of exposure appears crucial since prenatal exposure accounts for late VO (Salazar et al., 2004) whereas postnatal exposure is associated with normal timing (Gray et al., 2006; Moral et al., 2007) or early VO irrespective of the dose (Hu et al., 2013; Ma et al., 2006). Here, the complexity of neuroendocrine mechanisms appears since, in conditions causing early puberty, the arcuate nucleus expression of *Kiss1* mRNA and kisspeptin protein is increased but the receptor *GPR54* mRNA expression is reduced (Hu et al., 2013; Ma et al., 2006). Polybromi-nated EDCs appear to cause either no change or delay in VO depending on the dose and irrespective of the period of exposure (Stoker et al., 2004; Lilienthal et al., 2006; Ceccatelli et al., 2006). A similar conclusion can be drawn using DDT though there are no data after prenatal exposure. Using DDT during early postnatal life or after weaning, the dose appears critical for occurrence of early VO (Rasier et al., 2007; Heinrichs et al., 1971). Some of the mechanistic data that we have obtained in the hypothalamus after early postnatal exposure to DDT (Rasier et al., 2008,2007) are illustrated in Fig. 9. Both the age at VO and first estrus are advanced though using the highest DDT dose, time between VO and 1st estrus is increased and 1st estrus is not significantly advanced.

Such an observation has been thought to involve disorders of the central mechanism of ovulation since permanent estrus was seen subsequently. As already discussed, the reduced levels of serum LH could involve either negative feedback effect of DDT at the pituitary level as well as developmental changes or both. The pattern of GnRH secretion *in vitro* in the presence of DDT and antagonists of ER and the orphan dioxin AhR indicates involvement of both types of receptors in DDT effects.

As discussed above, distortion has appeared to be a component of the distribution of timing of pubertal events in humans with possible involvement of environmental chemicals. We have attempted to assess possible distortion of pubertal timing distribution based on rodent data when centiles for age at VO or BPS were available in the publication. In the study of Fernandez et al. (2010) where BPA causes early VO, distortion of pubertal timing towards lateness was seen versus controls. This was also observed to a lesser extent when early VO was caused by DES (Ashby et al., 1997). In contrast, when age at VO was delayed after DES exposure (Franssen et al., 2013), distortion of pubertal timing towards earli-ness was seen versus controls. Because the variance of pubertal timing is much less in rodents than in humans (Fig. 2), it is possible that variations in distortion are less likely to be observed in this animal model.

4. Summary and conclusion

Puberty results from the neuroendocrine activation or reactivation of the complex hypothalamic-pituitary-gonadal machinery. Quite remarkably, the timing of puberty shows important differences between individuals. After a constant advance between 1850 and 1960, it appears that, in several countries with relatively stable and uniform standard of life, menarcheal age has shown only minor progression during the last decades while breast development seems to still be advancing. Detailed analysis reveals that the pattern of age distribution is affected both in boys and girls. This leads to revise the belief that current changes in pubertal timing are prominently advancement in females. Current variations in pubertal timing involve few or no change in median age but a trend towards negative or positive distortion for initial or final pubertal stages, respectively, both in girls and boys.

Environmental factors can influence pubertal timing and reproduction through central effects that can be opposing and involve different mechanisms depending whether they take place early, during fetal and neonatal life or late during prepubertal life. As summarized in Table 10, exposure to sex steroids, insufficient energy availability, stress or endocrine disrupting chemicals can lead to different effects whether the exposure takes place during prenatal/early postnatal life or closer to puberty onset. Human and animal data suggest divergent effects of fetal/neonatal exposure to insufficient energy availability. While some human studies have shown that low birth weight is associated with early puberty, fetal/neonatal food restriction in rodents appears to delay the onset of puberty. Exposure to stress prenatally or peripubertally leads to a delayed onset of puberty in rodents. Again, human and rodents data appear to diverge regarding the early effects of stress. Early postnatal stress delays puberty in rodents while it seems to advance it in human. The effects of EDCs on pubertal timing are real but not univocal. Interpretation of the effects of exposure in the late prepubertal period is made more complex by the possible coexistence of effects at peripheral tissues and hypothalamic-pitu-itary levels. A peripheral effect lasting until puberty is less likely after fetal or neonatal exposure and reinforces involvement of neuroendocrine

mechanisms. Estrogenic EDCs, for instance, could directly affect vaginal opening or breast development by acting at the level of the vagina epithelium or the breast tissue, respectively. An effect lasting until puberty after fetal or neonatal exposure is less likely to be caused by a peripheral action and reinforces the likelihood of neuroendocrine mechanisms. Recent studies have shown that early postnatal exposure to EDCs could lead to dose-related opposing effects on pubertal timing. Knowing that EDCs such as BPA or phthalates can alter gene methylation, one can speculate that such early effects could be observed at the level of the GnRH system. Overall, the role of the neuroendocrine system appears to be prominent but needs integration with the peripheral effects and mechanisms. Low-dose mixtures of chemicals that represent the most common human and wildlife exposure, could have complex effects that have justified recent initiation of specific studies. Taking those data together, a revised paradigm of current changes in pubertal timing (Fig. 10) involves direct effect of several environmental factors including EDCs as well as interactions of such factors with gene expression through epigenetics. These mechanisms can take place in several age windows between fetal life and puberty. Reproductive maturation can be affected both directly and indirectly through changes in the control of energy balance. As a consequence in both sexes, it is possible that not only initial phases of pubertal maturation tend to occur earlier but final phases tend to occur later. Such an interpretation deserves longitudinal studies.

An additional lesson is that, in a causal perspective, the focus on a given adverse condition such as exposure to chemicals warrants to be complemented by an evaluation of consequences of combined adverse conditions. This may be crucial in areas around the world where nutritional repletion is rarely achieved in fetal life. Further, dissecting the effects of any adverse condition into closed areas like reproduction or energy balance may be artificial given the close links between those areas as far as both causality and effects are concerned. Finally, when adverse conditions starting even before conception are lasting for the entire prepubertal life, absence of effects does not mean that there is adaptive "compensation" and thus no harm. It means that two opposing effects are masking the reality of harm. Those concepts may have implications for clinicians, scientists in different disciplines (epidemiology, toxicology, endocrinology, public health, ...) and policymakers, for instance when it comes to define what is a suitable screening for adverse effects of exposure to possibly harmful conditions.

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