

Title	Early-life stress leads to sex-dependent changes in pubertal timing in rats that are reversed by a probiotic formulation.
Authors	Cowan, Caitlin S. M.;Richardson, Rick
Publication date	2018-07-24
Original Citation	Cowan, C. S. M. and Richardson, R. 'Early-life stress leads to sex-dependent changes in pubertal timing in rats that are reversed by a probiotic formulation', <i>Developmental Psychobiology</i> , In Press, doi: 10.1002/dev.21765
Type of publication	Article (peer-reviewed)
Link to publisher's version	<a href="https://onlinelibrary.wiley.com/doi/abs/10.1002/dev.21765">https://onlinelibrary.wiley.com/doi/abs/10.1002/dev.21765</a> - 10.1002/dev.21765
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Download date	2024-03-03 14:39:06
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**Early-life stress leads to sex-dependent changes in pubertal timing in rats that are reversed by a probiotic formulation**

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**Running Title:** Probiotics & Puberty after Early-Life Stress

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Puberty marks the beginning of a period of dramatic physical, hormonal, and social change. This instability has made adolescence infamous as a time of ‘storm and stress’, and it is well-established that stress during adolescence can be particularly damaging. However, prior stress may also shape the adolescent experience. In the present series of experiments, we observed sex-specific effects of early-life maternal separation stress on the timing of puberty onset in the rat. Specifically, stressed females exhibited earlier pubertal onset compared to standard-reared females whereas stressed males matured later than their standard-reared counterparts. Further, we demonstrated that a probiotic treatment restores the normative timing of puberty onset in rodents of both sexes. These results are in keeping with previous findings that probiotics reverse stress-induced changes in learned fear behaviors and stress hormone levels, highlighting the remarkable and wide-ranging restorative effects of probiotics in the context of early-life stress.

**Key words:** early-life stress; puberty; microbiota-gut-brain axis; probiotic treatment; maternal separation; development; rodents; rats

After the perinatal period, the most rapid changes in physical maturation occur at puberty (Rogol, Roemmich, & Clark, 2002). The transition into adolescence is not only a physical milestone but is also a period of vulnerability to mental health problems. Indeed, the peak period for the onset of many psychiatric disorders occurs during adolescence (Lee et al., 2014; Paus, Keshavan, & Giedd, 2008). In addition, the timing of puberty has been identified as a critical factor in mental health, with deviations from normative development placing an individual at greater risk for psychopathology (for reviews, see Mendle & Ferrero, 2012; Mendle, Turkheimer, & Emery, 2007; Waylan & Wolke, 2004; Weichold, Silbereisen, & Schmitt-Rodermund, 2003). There is extensive evidence that early pubertal maturation is linked to greater levels of anxiety, depression, eating pathology, and risky or anti-social behavior in girls (Belsky, Ruttle, Boyce, Armstrong, & Essex, 2015; Caspi, Lynam, Moffitt, & Silva, 1993; Mendle et al., 2007; Reardon, Leen-Feldner, & Hayward, 2009; Sun et al., 2016). For boys, the research has been more limited but there is evidence to support the suggestion that deviations from the normative timing (i.e., either early or late maturation) are associated with negative outcomes such as substance abuse, disruptive behavior disorders, and increased symptoms of depression (Graber, Seeley, Brooks-Gunn, & Lewinsohn, 2004; Mendle & Ferrero, 2012; Sun et al., 2016; Weichold et al., 2003). Of course, the correlational nature of these studies means that such shifts in pubertal timing may actually be a consequence of poor mental health (a possibility supported by at least one study that found anxiety at 8 years of age to be predictive of early menarche; Tremblay & Frigon, 2005) or a secondary symptom of some other common causal factor(s).

One of the shared risk factors for both psychopathology and deviations in pubertal timing is exposure to early-life stress. A number of studies have shown that stress accelerates pubertal development in girls, as well as in female rodents (Belsky et al., 2015; Li et al., 2014; Semiz, Kurt, Kurt, Zencir, & Sevinç, 2009; Tremblay & Frigon, 2005; but see also

Lau, Klinefelter, & Cameron, 1996). For example, there is a particularly strong association between childhood sexual abuse and early menarche (Boynton-Jarrett et al., 2013; Mendle, Leve, Van Ryzin, Natsuaki, & Ge, 2011; Mendle, Ryan, & McKone, 2016), but precocious puberty onset has also been observed in females exposed to less extreme cases of stress, such as low socio-economic status or low levels of maternal care (Borrow, Levy, Soehngen, & Cameron, 2013; Cameron et al., 2008; James-Todd, Tehranifar, Rich-Edwards, Titievsky, & Terry, 2010). For males, there has again been less research on the consequences of stress for puberty onset but the current literature suggests that physical maturation is altered in those exposed to early-life stress. The direction of the effects in males has been inconsistent, with some studies reporting accelerated maturation (e.g., Biagini & Pich, 2002; Sheppard & Sear, 2012) while others report delayed maturation (e.g., Bodensteiner, Christianson, Siltumens, & Krzykowski, 2014; Hernández-Arteaga et al., 2016; Semiz et al., 2009), perhaps due to the difficulties in measuring puberty onset in boys or the differences in the types and timing of stressors studied. Considering that shifts in either direction have been linked to problematic behavioral and psychological outcomes for males (as mentioned above), the impact of a shifted developmental trajectory may be important regardless of the direction of the shift. Taken together, this research suggests that stress can alter the timing of pubertal development for both males and females, which may in turn be either a mediator or a marker of increased risk for psychopathology.

Given the links between altered pubertal timing and psychopathology, the identification of treatments that prevent such shifts in puberty onset may be clinically relevant to reduce long-term mental health risk. In the present study, a gut-brain axis approach to this problem was assessed, targeting the microbiota (the collection of microorganisms residing within the gastrointestinal tract; Cowan, Callaghan, Kan, & Richardson, 2016a; Cowan et al., 2018; Foster, Rinaman, & Cryan, 2017). Recent work has

shown that sex differences in the microbiota emerge around puberty and the composition of the microbiota affects levels of key sex hormones such as testosterone (Markle et al., 2013). Preliminary evidence suggests that the microbiota of adult humans is also impacted by a history of early-life stress, with the relative abundance of specific microbial taxa correlating to self-reported history of early trauma (Labus et al., 2017). Supporting this correlational evidence, experimental induction of early-life stress has been shown to lead to alterations in microbiota composition across a range of animal models (e.g., Bailey et al., 2011; Gareau, Jury, MacQueen, Sherman, & Perdue, 2007; O'Mahony et al., 2009). Past work has demonstrated that probiotic treatments attenuate these effects of early-life stress on the microbiota, as well as attenuating stress-induced changes to hormonal and behavioral outcomes in developing individuals (Callaghan, Cowan, & Richardson, 2016; Cowan, Callaghan, & Richardson, 2016b; Desbonnet et al., 2010; Gareau et al., 2007). For example, a commercially available formulation of *Lactobacillus (L.) rhamnosus* and *L. helveticus* has been shown to reduce the corticosterone response of infant rats exposed to maternal separation stress (Gareau et al., 2007). The same probiotic formulation also restored developmentally appropriate fear-related behaviors (i.e., infantile amnesia and relapse-resistant extinction) in maternally-separated infant rats (Cowan et al., 2016b), whereas untreated maternally-separated infants exhibit accelerated maturation in this domain (i.e., adult-like extended fear retention and relapse-prone extinction; for a review, see Callaghan & Richardson, 2013).

The present experiments were designed to assess whether the effects of stress on pubertal timing can be similarly moderated by probiotic treatment. Physical markers of puberty onset were assessed in rats exposed to early-life maternal separation stress with or without probiotic treatment. Based on the research described above, it was hypothesized that maternal separation would shift the developmental trajectory of puberty, possibly in a sex-

dependent manner. To our knowledge, no published research has yet examined the effects of probiotic treatment on pubertal timing. However, based on previous studies demonstrating the stress-attenuating effects of probiotics (and specifically the *L. rhamnosus* / *L. helveticus* formulation) on behavioral, hormonal, and gastrointestinal outcomes, it was predicted that probiotic treatment would restore the normative developmental trajectories for puberty onset in both sexes.

## **Materials and Methods**

### **Subjects**

Experimentally naïve Sprague Dawley-derived rats were bred and housed in the School of Psychology at The University of New South Wales (original stock obtained from Animal Resources Centre, Western Australia). Rats were housed with their dam and littermates (culled to 8 pups per litter) until weaning. Rats were weaned between P21-23 and housed in same-sex groups (2-8 rats) that had been exposed to the same rearing condition. Animals were maintained on a 12-h light/dark cycle (lights on at 0700) with food and water available *ad libitum*. A maximum of 2 animals from the same litter were used in any given group. Where 2 rats of the same sex from the same litter were included the data was averaged and treated as a single data point for the statistical analyses (Cowan & Richardson, 2018). All animals were treated in accordance with *The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition* (2004), and all procedures were approved by the Animal Care and Ethics Committee at The University of New South Wales.

### **Maternal Separation**

During maternal separation (P2-14) all pups were removed from the home cage each day, weighed, and placed in an incubator as a litter for three hours (commencing between 0800 and 1200). The ambient temperature was maintained at approximately 27°C by a heat pad and 3 cm of bedding was provided so pups could behaviorally thermoregulate as needed.

## Probiotic Treatment

The probiotic treatment was a commercially available powder formulation, Lacidofil® (*Lactobacillus rhamnosus* R0011, 95%, and *Lactobacillus helveticus* R0052, 5%), provided by Lallemand Health Solutions (Montreal, QC, Canada). This particular formulation has been used in a number of published studies (for a review, see Foster, Tompkins, & Dahl, 2011) and was chosen based on previous findings that it normalizes corticosterone levels and fear regulation behavior in maternally-separated infants (Cowan et al., 2016b; Gareau et al., 2007). Furthermore, we have previously shown that administration of this formulation in the maternal drinking water leads to direct exposure of offspring to the probiotic species (Cowan et al., 2016b). Powdered Lacidofil was rehydrated in distilled water at a concentration of  $10^9$  CFU/mL and provided in dams' drinking water from P2-14 (i.e., the same period as maternal separation). Probiotic drinking solutions were changed every second day to ensure bacteria viability.

## Assessment of Puberty Onset

For females, puberty onset was assessed by vaginal opening (Parker & Mahesh, 1976). From P28 until vaginal opening, animals were weighed and briefly handled, placed against the body of the experimenter and the tail lifted in order to assess the vagina as closed or open. Group designation was known to the experimenter in Experiment 1a, but vaginal opening was determined by an experimenter unaware of the experimental condition of each rat in Experiment 1b.

For males, preputial separation was used as the measure of puberty onset and was defined by detachment of the prepuce, also known as the foreskin, from the glans penis (Korenbrodt, Huhtaniemi, & Weiner, 1977). Animals were observed every morning from P36 until preputial separation occurred. After weighing and brief handling, gentle pressure was applied to determine whether manual retraction of the prepuce to expose the glans penis was



possible (as is the case when separation has occurred; Korenbrot et al., 1977). All observations were carried out by an experimenter unaware of the experimental condition of each rat.

### Exclusions and Statistics

One rat from the standard-reared (SR) group (5.47 standard deviations [SD] from the mean age of puberty onset) and one rat from the maternally-separated (MS) group (3.83 SD from the mean) were defined as statistical outliers in Experiment 2 and were therefore excluded from the statistical analysis. No other exclusions were made.

All statistical analyses were performed with IBM Statistical Package for the Social Sciences (SPSS, v. 23). For t-tests, adjusted *t*-statistics and nominal *df* are reported when Levene's test for equality of variances was significant. Normality was assessed using the Shapiro-Wilk test and appropriate non-parametric tests were carried out when data were not normally distributed. This did not change the results of any analyses so, for consistency across all experiments, the parametric statistics are reported. For all analyses, *p* values less than .05 were considered statistically significant. Cohen's *d* was calculated using the equation  $d = (M_2 - M_1) / SD_{\text{pooled}}$  where  $SD_{\text{pooled}} = \sqrt{(SD_1^2 + SD_2^2) / 2}$ .

## Results

### Experiment 1: Puberty Onset in Stressed Females.

A history of early adversity has been linked to precocious puberty onset in female humans and rodents (Li et al., 2014; Mendle et al., 2011). Experiment 1 was comprised of two related experiments designed to replicate these findings in the maternal separation model of early-life stress and to assess the effects of a probiotic treatment administered during the period of stress. In Experiment 1a, the timing of physical pubertal onset (vaginal opening) was assessed in standard-reared (SR, *n* = 20) and maternally-separated (MS, *n* = 19) female

rats. In Experiment 1b, a probiotic-treated MS group (MS-Pro) was also included, leading to a 3-group design: SR ( $n = 20$ ), MS ( $n = 24$ ), and MS-Pro ( $n = 17$ ). In addition, animal weight was measured in Experiment 1b as an index of overall physical growth, which might be a contributing factor to MS-induced differences in pubertal timing (previous studies have shown that nutritional intake and body weight can influence pubertal timing in females, with timing of vaginal opening being more closely related to body weight rather than chronological age; Kennedy & Mitra, 1963).

**Vaginal opening.** The results of Experiment 1 are presented in Figure 1. In Experiment 1a, MS females exhibited vaginal opening at an earlier age than SR females (see Figure 1a). This was confirmed by an independent-samples t-test,  $t_{37} = 4.23$ ,  $p < .001$ ,  $d = 1.35$ . These results replicate past findings that puberty onset is accelerated in female humans and rats exposed to early-life stress (e.g., Li et al., 2014; Mendle et al., 2011). In Experiment 1b, the age of females' puberty onset also differed depending on the early rearing environment (see Figure 1b). Specifically, untreated MS females exhibited earlier vaginal opening compared to either SR or probiotic-exposed MS females. The statistical analysis confirmed this description; a one-way ANOVA indicated a significant effect of rearing condition,  $F_{2,58} = 4.77$ ,  $p = .012$ . Follow-up comparisons using the Student-Newman-Keuls (SNK) test indicated that MS females were significantly different to both SR females,  $p < .05$ ,  $d = .72$ , and MS-Pro females,  $p < .05$ ,  $d = .94$ , but these two groups did not differ from each other,  $p = .74$ ,  $d = .10$ .

**Weight.** Changes to the timing of pubertal onset did not occur as a result of differences in the weight of animals across conditions. Using weight data collected in Experiment 1b (which included all three rearing conditions), there was no effect of rearing condition on weight at P30,  $F < 1$  ( $M_{SR} = 106.6 \pm 1.8g$ ,  $M_{MS} = 103.3 \pm 1.8g$ ,  $M_{MS-Pro} = 106.9 \pm 3.1g$ ). Further, there was no correlation between weight at P30 and age of vaginal opening

for the overall sample,  $r_{58} = .07$ ,  $p = .63$ , or for the individual groups, largest  $r_{20} = .17$ ,  $p = .47$  (data not shown).

Together, the results of Experiment 1 provide two replications of past findings that stress accelerates pubertal onset in females. More importantly, Experiment 1b demonstrated for the first time that a probiotic treatment prevents this precocious physical maturation in stressed females. This novel finding is in keeping with previous work showing that the same probiotic formulation can prevent stress-induced shifts in the trajectories of fear memory and extinction development across generations (Callaghan et al., 2016; Cowan et al., 2016b).

### **Experiment 2: Puberty Onset in Stressed Males.**

The results of Experiment 1 add to the existing evidence that stress accelerates pubertal development in females (e.g., Mendle et al., 2011). In addition, Experiment 1b established that a probiotic treatment can restore the normative timing of pubertal onset in these stressed females. Previous research suggests that stress also alters the developmental trajectory of puberty for males, although as noted earlier the direction of this effect has been inconsistent (e.g., Semiz et al., 2009; Sheppard & Sear, 2012). The aim of Experiment 2 was to examine the effects of both maternal separation and probiotic treatment on pubertal timing and body weight in male rats, using preputial separation as a physical marker of puberty onset (Korenbrot et al., 1977). A between-subjects design was used with three groups that differed in their early rearing environment (SR:  $n = 16$ , MS:  $n = 22$ , and MS-Pro:  $n = 16$ ).

**Preputial separation.** The age of puberty onset in males differed depending on early rearing environment (see Figure 2a). MS males exhibited delayed onset of preputial separation compared to SR males, but this effect of stress was eliminated by exposure to probiotics. The statistical analysis confirmed this description; a one-way ANOVA indicated a significant effect of rearing condition,  $F_{2,51} = 7.77$ ,  $p = .001$ . Follow-up comparisons using the SNK test indicated that preputial separation occurred significantly later in MS males

compared to both SR males,  $p < .05$ ,  $d = 1.14$ , and MS-Pro males,  $p < .05$ ,  $d = 1.07$ , but the SR and MS-Pro groups did not differ from each other,  $p = .66$ ,  $d = .13$ . These results provide a replication of previous findings that stress can delay pubertal onset in MS male rats (Bodensteiner et al., 2014). More importantly, they demonstrate for the first time that a probiotic treatment can prevent the effects of stress on pubertal development in male rats.

**Weight.** In this experiment, the MS males were lighter at the start of the puberty inspection period (P37;  $M = 168.9 \pm 1.9\text{g}$ ) compared to the SR ( $M = 182.2 \pm 4.2\text{g}$ ) and MS-Pro males ( $M = 183.6 \pm 5.2\text{g}$ ). This effect was statistically significant,  $F_{2,51} = 5.29$ ,  $p = .008$ , with follow-up SNK comparisons showing that the MS males were significantly lighter than both SR and MS-Pro males,  $p < .05$ , whereas these two groups did not differ from each other,  $p = .80$ . Moreover, the age of physical puberty onset was negatively correlated with weight such that lower initial weight was associated with later preputial separation,  $r_{56} = -.62$ ,  $p < .001$  (see Figure 2b). This relationship between weight and age of puberty onset, corresponding to a large effect size, was similar for rats of all rearing histories (SR:  $r_{17} = -.60$ ,  $p = .01$ ; MS:  $r_{23} = -.68$ ,  $p < .001$ ; MS-Pro:  $r_{16} = -.56$ ,  $p = .02$ ).

Together with the results of Experiment 1, these findings reveal two interesting gender dissociations for the effects of stress on pubertal timing. First, the timing of puberty onset was shifted in opposite directions for stressed males and females. Second, the effect of rearing condition on pubertal timing was related to differences in pre-pubescent weight for males only. The effect of rearing condition on body weight in our male subjects was unexpected as we have previously found no effect of maternal separation or probiotic treatment on infants' weight (Callaghan & Richardson, 2011; Cowan et al., 2016b). However, these pre-pubescent weight differences may provide an explanation for the delayed puberty onset observed in stressed males. Previous studies have identified that late puberty onset encourages growth, allowing smaller individuals to “catch up” to their larger peers (Wit,

Visser-van Balen, Kamp, & Oostdijk, 2004). In keeping with this hypothesis, the relationship between weight and pubertal timing was observed across all three groups. Furthermore, we have collected weight data in separate studies from two independent cohorts of experimentally-naïve adult males which shows that MS males do indeed recover to normal weight by adulthood (see Table 1; largest  $t_{36} = .64$ ,  $p = .53$ ).

## Discussion

The present experiments were designed to assess the impact of different early rearing conditions on physical maturation at the onset of puberty in both male and female rats. The results show that maternal separation during early infancy alters the timing of puberty onset in a sex-dependent manner, leading to precocious pubertal development in female rats but delayed pubertal maturation in males. These findings are consistent with the existing literature on pubertal development in humans, where the most commonly reported effects of stress are accelerated puberty onset in stressed females and “off-time” (i.e., either accelerated or delayed) puberty onset in stressed males (e.g., Mendle et al., 2011; Negri, Blankson, & Trickett, 2015; Semiz et al., 2009; Sheppard & Sear, 2012). Furthermore, these experiments provide the first evidence that a probiotic treatment can restore the normative developmental timing of puberty onset following stress. The results were equally striking for female and male rats; across both sexes, probiotic treatment of stressed rats resulted in complete restoration of the timing of pubertal onset to the ages observed in unstressed rats. This demonstrates that the stress-attenuating effects of this probiotic formulation extend beyond infancy and beyond emotion regulation (Callaghan et al., 2016; Cowan et al., 2016b), with exciting implications for the long-term benefits of this treatment. It should be noted that given our interest in whether probiotics might ameliorate the effects of early life stress on pubertal timing we only examined animals exposed to maternal separation in this study. That

is, we do not know the effects of the probiotics on pubertal timing in SR animals, and that is something that future work should explore.

From a theoretical standpoint, it is worth considering the implications of the current results for the stress acceleration hypothesis (Callaghan & Tottenham, 2016a, 2016b). While this theory posits that maturation is accelerated by early adversity, the authors explicitly limit this proposal to the accelerated maturation of emotion-relevant brain circuits and associated behaviors. Indeed, Callaghan and Tottenham (2016a, 2016b) suggest that stress invokes a “reprioritization” of developmental trajectories that may accelerate emotional maturation at the expense of other brain regions or functions. This logic can be used to explain the contrast between the current results, which find *delayed* development in stressed males, and our prior work, which has consistently found development of fear-related behavior to be *accelerated* in stressed males and their offspring (Callaghan et al., 2016; Callaghan & Richardson, 2012a, 2012b, 2013; Cowan, Callaghan, & Richardson, 2013). That is, the early maturation of emotional behavior in male MS rats might be considered to reflect a greater allocation of resources to these functions that then reduces the resources available for physical development and slows puberty onset.

But how does one then account for the opposite effects of early-life stress on puberty onset in males and females? The type of “resource restriction”-induced delay suggested by the stress acceleration hypotheses might occur specifically in males if there were gender differences either in the resources required to instigate puberty or in the experienced severity of the early-life stressor. Past research suggests that extreme stressors (e.g., war, starvation) are more likely to delay puberty onset than accelerate it, even in females (Ellis, 2004). Further, there is some evidence that females are more resilient to the effects of maternal separation, at least on certain measures (Diehl et al., 2007; Kalinichev, Easterling, Plotsky, & Holtzman, 2002; Romeo et al., 2003). For example, Kalinichev et al. (2002) found that, even

though MS rats of both sexes exhibit increased anxiety on the elevated plus maze in adulthood, only MS males exhibit exaggerated startle responses to acoustic stimuli. Although the reasons for these gender dissociations remain unclear, the idea that male rodents are more sensitive to maternal separation is supported by the differential effects of maternal separation on pre-pubescent weights in the experiments presented here. That is, untreated MS males exhibited lower body weights compared to SR and probiotic-treated MS males, an effect that was not seen in the females. This result potentially hints at a harsher physical impact of the maternal separation stress on males.

Turning to a more mechanistic explanation for the sexual dimorphism observed in the current experiments, the influence of gonadal steroid hormones is an obvious starting point. These hormones, including testosterone and estrogen, are produced by the hypothalamic-pituitary-gonadal (HPG) axis and are not only involved in sexual behavior and the onset of puberty but also in the sexual differentiation of neural circuits and immunological functions (Morale et al., 2001). Though the HPG axis incorporates different sub-nuclei of the hypothalamus and pituitary compared to the stress-regulating hypothalamic-pituitary-adrenal (HPA) axis, these two neuroendocrine pathways are closely linked in both physical proximity and function, making it unsurprising that the HPG axis is sensitive to stress (Dismukes, Johnson, Vitacco, Iturri, & Shirtcliff, 2015). For example, low levels of testosterone have been reported in both male and female rodents exposed to stressful rearing environments. In MS males, delayed preputial separation is accompanied by low testosterone in the peri-pubertal period (Bodensteiner et al., 2014). The female offspring of low licking-grooming mothers, which exhibit accelerated puberty onset (similar to MS females in the current experiments), also exhibit low testosterone levels (measured prenatally; Cameron et al., 2008). Even though testosterone levels were measured at very different time-points in

those two studies, the data hint at a sex-specific role for gonadal hormone exposure in regulating stress-induced alterations to pubertal timing.

Our understanding of the interactions between the microbiota and the HPG axis is currently quite limited, despite clear links between the microbiota and HPA axis regulation (de Weerth, 2017; Rea, Dinan, & Cryan, 2016; Sudo, 2014). For example, germ-free rodents, which are raised in a completely sterile environment to prevent colonization of the microbiota, exhibit exaggerated ACTH and corticosterone stress responses (Clarke et al., 2013; Sudo et al., 2004). In addition, certain probiotics (including the current formulation) have been shown to dampen the corticosterone response to stress, mostly in rodents but in at least one study of healthy humans (Ait-Belgnaoui et al., 2012; Bravo et al., 2011; Gareau et al., 2007; Messaoudi et al., 2011). The vagus nerve has been shown to be instrumental for such microbial modulation of the HPA axis (Bravo et al., 2011), although undoubtedly this is one of multiple routes of communication alongside other microbiota-gut-brain pathways (e.g., the immune system and bacterial metabolite/neurotransmitter production). Microbiota-HPA axis communication is likely to have implications for the function of the HPG axis, particularly under conditions of early-life stress, where it has been shown that the HPA and HPG axes become more tightly coupled (Dismukes et al., 2015).

Indeed, there is some evidence in the literature that the microbiota can regulate gonadal hormone production. It is now well established that there are gender differences in the composition of the gut microbiota (e.g., Haro et al., 2016; Org et al., 2014), but the impact of these microbial gender differences on hormone production and other outcomes has only recently been revealed. Research conducted by two separate groups (Markle, Frank, Adeli, von Bergen, & Danska, 2014; Markle et al., 2013; Yurkovetskiy et al., 2013) demonstrated that transfer of the gut microbiota from male mice into females increases serum testosterone levels and protects against autoimmune problems (which are more prevalent in



females). In light of these studies, it might be hypothesized that stress-induced disruption of the composition of the microbiota alters levels of gonadal hormones. This would suggest that the protective effects of the probiotic treatment in the current studies might be the result of probiotics normalizing HPG axis function and hormonal levels **through normalization of the stressed gut microbiota**. Further investigations to measure or manipulate gonadal steroids in both sexes (either directly or perhaps via fecal microbiota transplant) would be useful to identify the developmental trajectories of these hormones and their role in regulating the effects of stress and probiotic treatment.

The gender specificity of the effects of stress on physical maturation highlights the importance of studying the impact of a given manipulation in both females and males. Unfortunately, **many areas of psychological research, particularly preclinical studies of the neurobiological substrates of psychological processes, have traditionally focused** solely on males despite the disproportionate numbers of women affected by common psychological disorders such as anxiety and depression (Altemus, Sarvaiya, & Epperson, 2014; Li & Graham, 2017). The gender specificity of the current findings raises the question of whether similar sex differences would be observed in previously reported findings on the performance of stressed infants in terms of behavioral fear regulation. For example, if females are indeed more resilient to the effects of maternal separation, then it may be the case that the effects of stress on the development of long-term fear retention and fear relapse would be attenuated in females when compared to the clear shifts observed in MS males (Callaghan et al., 2016; Callaghan & Richardson, 2013). Regardless, the results reported here support the extant literature regarding the disruptive effects of early-life stress and provide novel evidence to strengthen the argument for the potential of probiotic treatments to confer beneficial effects in stressed populations.

## Notes

### Acknowledgements

This work was supported by grants from the Australian Research Council (DP150104835) and the National Health and Medical Research Council (APP1031688) to R.R., as well as a Petre Foundation scholarship and a UNSW Research Excellence Award to C.C. The authors thank Dr. Thomas Tompkins and Jocelyn Belvis at Lallemand Health Solutions (Montreal, QC, Canada) who provided the Lacidofil<sup>®</sup> gratis.

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## Tables

**Table 1** Mean Weights of Adult Male Rats with Different Early Rearing Histories.

Cohort	Rearing Condition	n	Mean ( $\pm$ SEM)	
			Age (days)	Weight (g)
1	Standard-reared	22	63.3 (0.37)	385.0 (7.40)
	Maternally-separated	15	62.3 (0.73)	390.9 (13.98)
2	Maternally-separated	19	97.9 (2.06)	575.3 (13.63)
	Maternally-separated, probiotic-exposed	19	101.5 (4.31)	590.0 (18.60)

**Figure Captions**

**Figure 1.** Mean ( $\pm$  SEM) age of vaginal opening for (a) standard-reared (SR) and maternally-separated (MS) rats in Experiment 1a and for (b) SR, MS, and probiotic-exposed, maternally-separated (MS-Pro) rats in Experiment 1b. Vaginal opening was observed at an earlier age in MS females compared to SR or MS-Pro females.  $n = 17-24$ .  $*p < .05$

**Figure 2.** (a) Mean ( $\pm$  SEM) age of preputial separation for standard-reared (SR), maternally-separated (MS), and maternally-separated, probiotic-exposed (MS-Pro) male rats. MS males exhibited a delay in preputial separation compared to SR and MS-Pro males. (b) Weight at P37 correlated with age of puberty onset.  $n = 16-22$ .  $*p < .05$