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Oestrual Phase at first exposure to Predator-induced Stress Determines Pattern of Alterations in Oestrous Cycle and Endocrine Response in the Rat

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Summary: Stress has been acknowledged as one of the aetiologies of female reproductive dysfunction, yet the mechanisms involved are not totally elucidated. Based on the paucity of information on how predator-induced stress (PS) affects oestrous cycle in rats, this study was designed to investigate the effect of PS on the oestrous cycle in rats. Forty-eight (48) Sprague-Dawley rats were used for this study. They were randomly divided into Control and PS group. Each group was divided into four subgroups (n=6/group) according to the phases of oestrous cycle. Stress was induced by exposing rats to cat (predator) for 60 minutes/day for 14 consecutive days. PS caused significant disruption of the oestrous cycle. In animals subjected to PS at proestrus (PS-proestrus) and oestrus (PS-oestrus), percentage occurrence of proestrus, oestrus and metestrus phases were significantly reduced compared with control. In animals subjected to PS at metestrus (PS-metestrus) and diestrus (PS-diestrus), percentage occurrence of oestrus phase was not significantly affected. In all animals exposed to PS, percentage occurrence of diestrus was significantly increased regardless of the phase of first exposure compared with control. Corticosterone and prolactin levels were significantly elevated in PS groups compared with control. Progesterone was significantly increased in animals at diestrus phase compared with oestrus phase and respective phases in control. Oestradiol was significantly reduced in PS group compared with control at oestrus phase but not significantly different at diestrus phase. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels were significantly lower in PS groups at oestrus phase compared with diestrus phase. This study shows that PS disrupts the oestrous cycle secondary to perturbation of hormonal control of female reproduction and is influenced by the phase at first exposure to stress.

Keywords*:* Predator, Vaginal Cytology, Oestrous Phases, Prolactin, Corticosterone, Stress

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INTRODUCTION

Stress, which is biologically defined as disruption of homeostasis, has been acknowledged as one of the aetiologies of female reproductive dysfunction such as menstrual cycle disturbances, amenorrhea, and infertility (Xiao *et al.*, 2002; Li *et al.*, 2004; Pierce *et al.*, 2004). Although stress has been consistently reported to activate the hypothalamic- pituitary-adrenocortical (HPA) axis and disrupts the hypothalamic-pituitary-gonadal (HPG) axis, yet the mechanisms involved are not totally elucidated.

Historically, menstrual cycle disturbance as a result of exposure to stressful conditions was observed in British women interned in Japanese concentration camp in Hong Kong. In the said population, it was reported that 60% of the women developed amenorrhoea which persisted for several months (Sydenham, 1946; Sanders and Bruce, 1999). Since then attention has been focused on the impact of stress on reproductive health with the purpose of understanding how stress affects reproductive function. However, findings on the impact of stress on the menstrual cycle have been inconsistent (Sanders and Bruce, 1999; Clarvit, 1988; Allisworth *et al.*, 2007; Sood *et al.*, 2012). Recently, psychosocial stress as a result of conflict in Syria was associated with menstrual irregularities in Syrian refugee women in Lebanon (Masterson *et al.*, 2014). In the laboratory, animal studies reveal differential response to different stress model (Xiao *et al.*, 2002; Xiao *et al.*, 1998; Chatterjee and Chatterjee, 2009; Saraswathi *et al.*, 2012). Furthermore, a confounding factor in stress studies is that varying levels of ovarian steroids across phases of menstrual cycle may modulate the response to stress (Edozien, 2006). Surprisingly, investigations considering the phases of the oestrous cycle at first exposure to stress are scarce.

Predator-induced stress (PS), is an ethologically relevant stress model and affords an excellent means to resolve central mechanisms responsible for internally generated stress responses (Figueiredo *et al.*, 2003; Clinchy *et al.*, 2013). It is a natural stressor that is not painful and does not evoke reactive response to physiologic challenges (Figueiredo *et al.*, 2003). PS is likened to the experiences of: people living in war torn

region of the world, women in abusive relationships, or victims of rape. But in literature there are no reports, to the best of our knowledge, on the impact of predator-induced stress on female reproductive function.

Given the endemic nature of stress in our society (Kudielka and Wust, 2010), this study was therefore designed to investigate the effect of predator stress, a form of psychosocial stress, on the oestrous cycle in rats with emphasis to the phase of oestrous cycle at first exposure to stress.

MATERIALS AND METHODS

Forty-eight Sprague Dawley rats (160-180g) were used for this study. They were obtained from the Animal House, College of Medicine of the University of Lagos. Animals were housed in 3- compartment wooden cages under 12:12 h light-dark cycle (lights on at 06:00 h). They were allowed to acclimatize for two weeks before the commencement of the experiment and had access to food (normal rat chow - Livestock Feed Limited, Nigeria) and water *ad libitum*. Before the commencement of the experiment, daily vaginal smears of the rats were observed for 14 days to obtain 3 consecutive cycles. Animals that had 3 consecutive normal cycles were used for the study. The phase of oestrous cycle was determined according to the method of Marcondes *et al.*, 2002.

Animals were divided into Control and Predator-induced Stress (PS) groups (n=24/group). Animals in each group were further subdivided into four sub-groups according to the phase of the oestrous cycle at first exposure to stress i.e. CNTRL-proestrus, CNTRL-oestrus, CNTRL-metestrus and CNTRL-diestrus in control group and PS-proestrus, PS-oestrus, PS-metestrus and PS-diestrus phases in PS group (n=6/phase). Animals in the stress group were subjected to stress for 14 consecutive days. During this period the phases of the oestrous cycle were monitored. The percentage occurrence of each phase in a group was calculated as a fraction of the product of all phases and experimental duration in days multiplied by the number of animals in a group.

To induce stress in the PS group, a modified method of Figueiredo *et al.* (2003) was used. Cat (predator) was put in the middle compartment of the cage while rats to be stressed were housed in the compartments on either side of the cat compartment. This allows rats to have olfactory, visual, and auditory cues on exposure with predator but no physical contact. Before the commencement of stress exposure, the phase of oestrous cycle was noted after which animals were subjected to stress 60 minutes daily for 14 consecutive days between the hours of 08:00-09:00 or 09:00-10:00. On the last day of stress exposure, the phase of oestrous cycle was noted, and then animals were euthanized by cervical dislocation. Blood samples were collected by cardiac puncture into plain sample bottles and centrifuged at 3000rpm for 15 minutes to obtain serum for hormonal analysis. All assays were done using the enzyme-linked-immunosorbent assay (ELISA) techniques for the determination of corticosterone, prolactin, progesterone, oestradiol, LH and FSH levels in serum.

Statistical Analysis

Analysis of data was done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). Results are presented as mean ± standard error of mean (SEM). Differences between groups were analyzed by one-way ANOVA followed by Student's Newman-Keuls post-hoc test for multiple comparisons. Differences were considered significant when $p<0.05$.

RESULTS

Effect of PS on the oestrous cycle in rats

The percentage occurrence of proestrus, oestrus and metestrus phases were significantly ($p<0.05$) reduced in rats first exposed to predator at proestrus and oestrus phases compared with control respectively. In animals that began stress exposure at metestrus and diestrus phases respectively, percentage occurrence of oestrus phase was not significantly ($p>0.05$) affected but proestrus and metestrus phases were significantly ($p<0.05$) reduced. However, irrespective of the phase at first exposure to stress, diestrus phase was significantly prolonged (Figure 1a-1d).

The pattern of oestrous cycle is shown in figure 2-3. The oestrous cycle was significantly disrupted in animals subjected to PS. Animals in PS-proestrus, after completion of the first cycle, stayed at diestrus (about 8-9 days) only to resume the cycle around day 9 or 10 of the oestrous cycle. The average number of occurrence of oestrus phase (2.2±0.17) was significantly ($p<0.05$) lower compared with CNTRL-proestrus (4.0±0.0). Animals in PS-Oestrus also experienced disruption with significant reduction in average number of occurrence of oestrus phase (2.5±0.43) compared with CNTRL-oestrus (3.7±0.42).

Effect of PS on plasma concentration of corticosterone, prolactin, oestradiol and progesterone during the oestrous cycle

Figure 4 (a-d) shows the effect of PS on serum level of corticosterone, prolactin, oestradiol and progesterone. Corticosterone values are shown in Fig. 4a. Post hoc Student Newman-Kuels test revealed that corticosterone concentration was significantly ($p<0.05$) higher in PS animals sacrificed at oestrus and diestrus phases compared with corresponding phases in control. Prolactin concentration was higher in PS animals compared with control animals ($p<0.05$).

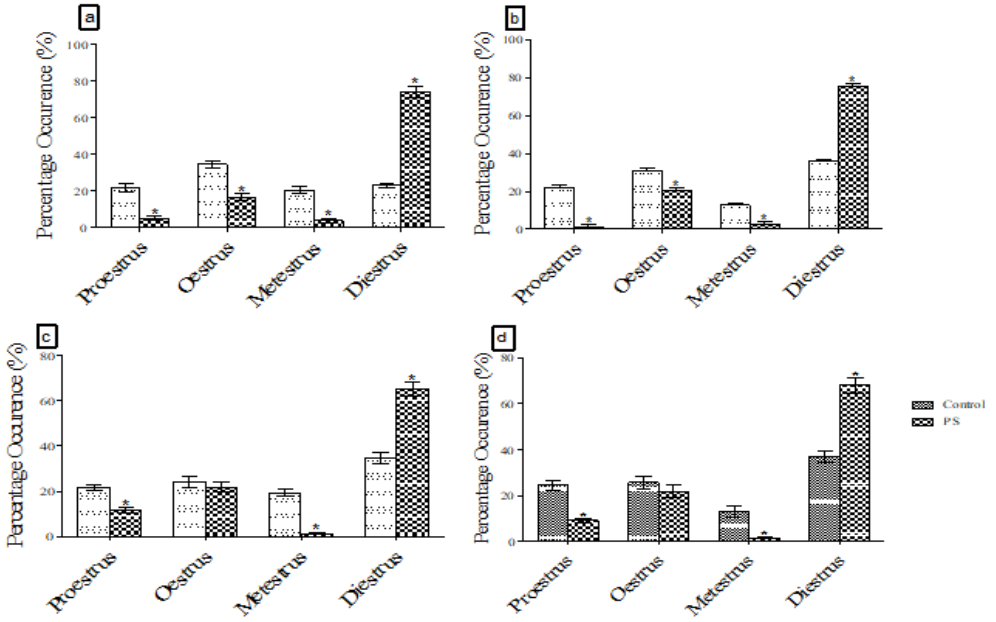

Figure 1: Percentage (%) occurrence of oestrous phases in animals that commenced exposure to PS in (a) proestrus (b) oestrus (c) metestrus and (d) diestrus phase of oestrous cycle. * indicates significant difference from control at $p<0.05$.

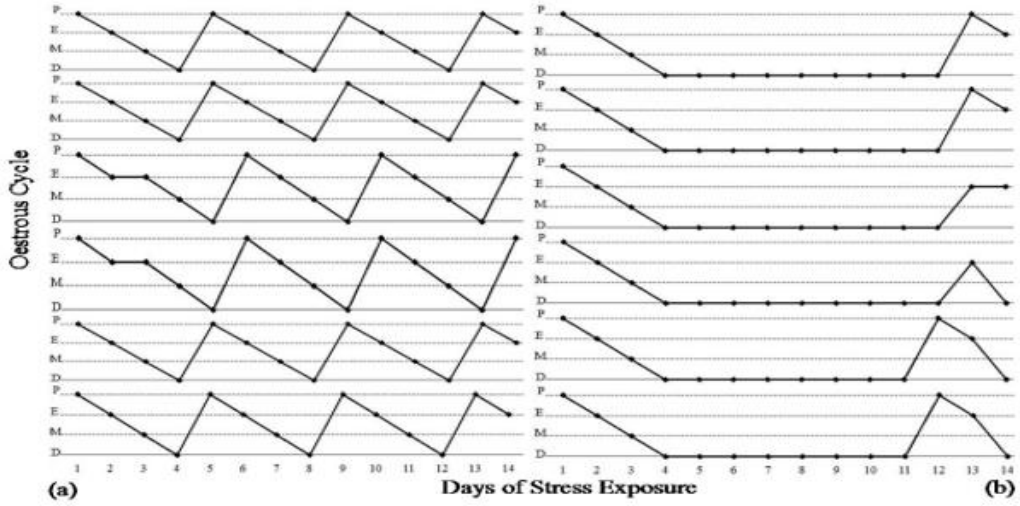

Figure 2: Pattern of Oestrous Cycle in (a) Control (CNTRL-proestrus) and (b) PS (PS-proestrus) animals starting at Proestrus. P = proestrus, E = oestrus, M = metestrus, and D = diestrus. Note the reappearance of proestrus phase in the stressed animals around day 11 and 12 of stress period after the cycle has been suspended at diestrus.

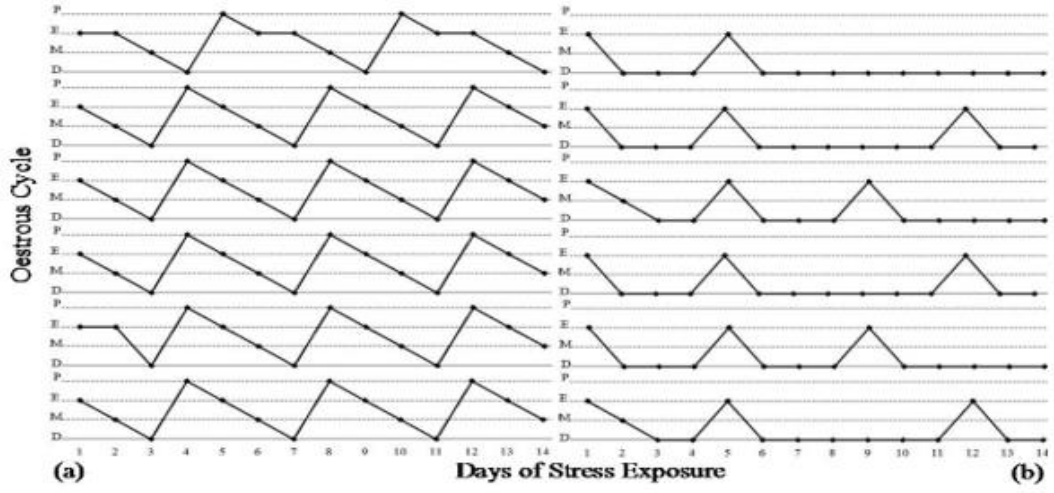

Figure 3: Pattern of Oestrous Cycle in (a) Control (CNTRL-oestrus) and (b) PS (PS-oestrus) animals starting at oestrus. P = proestrus, E = oestrus, M = metestrus, and D = diestrus.

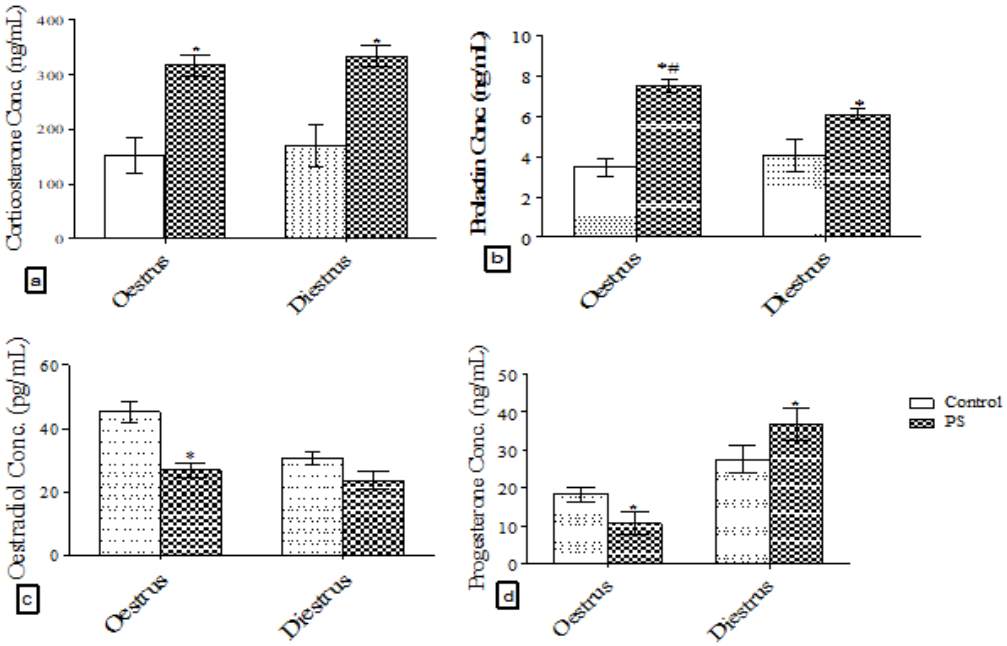

Figure 4: Effect of PS on serum (a) corticosterone (b) prolactin (c) oestradiol and (d) progesterone at oestrus and diestrus phases of the oestrous cycle. All animals were sacrificed only at oestrus and diestrus phases. * shows significant difference at $p<0.05$ compared with control; # shows significance compared with stress diestrus at $p<0.05$.

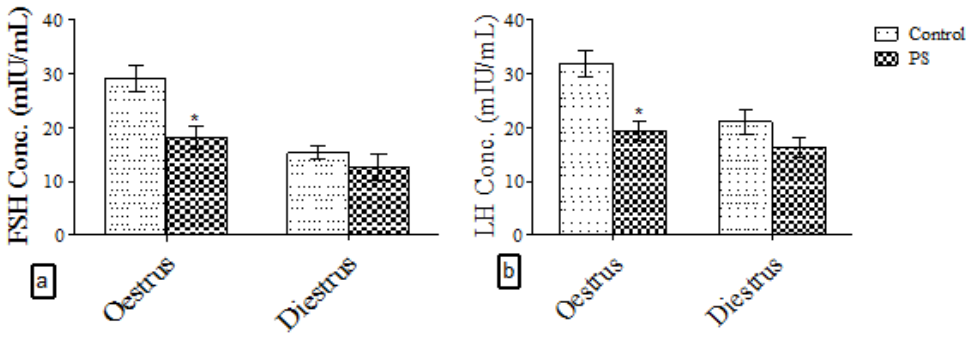

Figure 5: Effect of PS on serum (a) FSH and (b) LH at oestrus and diestrus phases of the oestrous cycle. All animals were sacrificed only at oestrus and diestrus phases. * indicates significant difference from control at $p<0.05$.

There was also significant ($p<0.05$) elevation of prolactin levels in PS animals at oestrus phase compared with diestrus phase (Fig. 4b). Oestradiol was significantly ($p<0.05$) reduced only in the stress oestrus phase compared with control but no significant difference compared with stress diestrus. Progesterone level (Fig. 4d) was higher in stress diestrus phase compared with control but significantly lower in stress oestrus phase compared with control ($p<0.05$. Note that at the end of the experiment animals stayed in oestrus and diestrus phases irrespective of the phase at first exposure to PS.

Effect of PS on plasma concentration of LH and FSH during the oestrous cycle

Fig. 5a shows the effect of PS on FSH concentration. Animals sacrificed at oestrus phase exhibited significant reduction in serum FSH when compared with control ($p<0.05$). Significant ($p<0.05$) reduction in serum LH was also observed at oestrus phase compared with control ($p<0.05$) (Fig. 5b). Note that at the end of the experiment animals stayed in oestrus and diestrus phases irrespective of the phase at first exposure to PS.

DISCUSSION

This study shows that PS caused significant disruption of normal cyclicity of the oestrous cycle in rat. In all the groups of animals subjected to PS, animals stayed on diestrus phase for well above 60% of the oestrous cycle while the metestrus phase was significantly reduced. However, in PS-proestrus and PS-oestrus groups, the percentage occurrence of proestrus and oestrus phases were significantly reduced compared with animals in PS-metestrus and PS-diestrus groups (Figure 1). This observation reveals that the pattern of stress-induced alteration of oestrous cycle of rats is dependent on the phase of the oestrous cycle at first exposure to stress. In this study, the most vulnerable phases to PS are the proestrus and oestrus phases. Also, observable in this study is the reoccurrence of proestrus phase on day 11 or 12 in animals that began exposure to PS at proestrus after the cycle have been suspended at diestrus for about 8 days.

The significant reduction in the percentage occurrence of proestrus phase in all the groups of stressed animals is an indication of psychological stress which can cause poor sexual receptivity in the female rat. This phase is the beginning of sexual receptivity and ensures timely fertilization of the released ova immediately after ovulation (Paccola *et al.,* 2013). The reduced occurrence of proestrus phase can also be as a result of shortening of the time length of this phase i.e. the period of this phase is reduced which can be possibly missed at the time of smearing and therefore cannot be accounted for. The implication of this is reduced time for follicular development (Maeda *et al.,* 2000; Paccola *et al.,* 2013) (proestrus phase = phase of follicular development) which can affect the number and quality of released ova after ovulation. Similarly, the reduced percentage occurrence of oestrus phase, especially in the PS-proestrus and PS-oestrus groups, shows that this stress model can shortened or prevent the fertile period in the rat. Oestrus phase is the period female mammals are on heat and are receptive to the male. It is also part of the follicular phase (together with proestrus) and ultimately leads to ovulation (Goldman *et al.,* 2007). Significant reduction of this phase (hence the fertile period or window) has been reported to be a serious reproductive health issue in the female and is most often the cause of infertility (Smeenk *et al.,* 2005; Louis *et al.,* 2011). Reduction in the percentage occurrence of metestrus (which is the beginning of luteal phase) in all the groups of stressed animals could also possibly be as a result of short length of this phase which is about 6- 8 hours (Hubscher *et al.,* 2005).

The negative impact of the stress model employed in this study is evident from the significant increase in serum corticosterone in stressed animals compared with control (Figure 4a). This shows that this stress model caused robust activation of the hypothalamic-pituitary-adrenal (HPA) axis. This is critical in stress response and confirms previous findings that PS is ethologically relevant in the study of stress and its impact on reproductive performance in rats (Figueiredo *et al.,* 2003). In this study, elevation of corticosterone was irrespective of the phase at first exposure to stress. Although elevated glucocorticoid had been argued to be necessary for stress response (Creel *et al.,* 2009), the result of this study confirms the reports of other investigators that elevated glucocorticoid is involved in the neuroendocrine response to stress (Jacobson and Sapolsky, 1991; Viau, 2002; Liu *et al.,* 2014).

Prolactin is another hormone often alluded to in literature to be involved in stress-response (Sobrinho, 2003; Donadio *et al.,* 2005; Insana and Wilson, 2008; Ranabir and Reetu, 2011). In this study, results revealed that there was significant elevation in prolactin level in stressed animals compared with control irrespective of the phase at first exposure (Figure 4b). Although normal cyclic increase in prolactin occurs during proestrus phase of the oestrous cycle and in some cases in the afternoon of oestrus (Furudate *et al.,* 1989; Freeman *et al.,* 2000), a high positive correlation has been shown to exist between prolactin and corticosterone secretion during stress (Gala, 1990; Torner, 2016). Our results, however, contradicts the report of López-Fontana *et al.* (2011) that elevated glucocorticoid prevented prolactin release. This is because the neuronal circuits involved in regulation of the physiological response to stress stimulate the HPA axis and consequently caused prolactin secretion (Bahgat, 2012). Also, there is increase in the expression of mRNA of immediate early gene c-fos – a marker of neuronal activation – in the cingulate cortex, hippocampus, and medial amygdale, which are areas that play important roles in the HPA response to stress (Figueiredo *et al.,* 2003). This has been reported to influence prolactin secretion during the oestrous cycle, pregnancy, and pseudopregnancy during stress (Polston and Erskine, 2001). Furthermore, the significant increase in serum prolactin may be part of an elaborate stress coping techniques. This is because prolactin has been reported to possess anxiolytic and anti-stress effects on the brain (Torner *et al.,* 2001, Torner, 2016). In addition, elevated prolactin has been reported to cause disruption in reproductive functions and might play a contributory role in the extended diestrus phase observed in the stressed animals (Grachev *et al.,* 2015).

Gonadal steroids are important for growth, development and function of female reproductive tissues (Gava *et al.,* 2004); and also play central role in ovulation and sexual behavior (Antunes *et al.,* 2006; Donadio *et al.,* 2005). In this study, progesterone secretion was significantly reduced in stressed animals sacrificed during oestrus phase compared with control while diestrus phase secretion was significantly increased in stressed animals compared with control. This phase observations may be as a result of reduced postovulatory secretion from luteinization of the follicular granulosa and thecal cells converting them to corpus luteum (Rodriguez-Echandia *et al.,* 1988). Furthermore, the significant increase in diestrus phase concentration of progesterone may be responsible for the sustained diestrus phase observed in stressed animals (Magiakou *et al.,* 1997; Antunes *et al.,* 2006).

Exposure to PS caused significant decrease in serum level of oestradiol only in animals sacrificed at oestrus phase of the oestrous cycle compared with control animals (Figure 4c) while there was no significant difference in oestradiol concentration in animals sacrificed in diestrus phase compared with corresponding phase in control. The decrease in oestradiol observed at oestrus phase may be as a result of depressed folliculogenesis due to significant elevation in glucocorticoid (Hsueh and Erickson,

1983). Glucocorticoid receptors have been discovered in the rat ovary and ovarian granulosa cell cytosol, activation of which can results to suppression of granulosa cell aromatase enzyme activity leading to oestrogen deficiency (Bakker and Baum, 2000; Chatterjee and Chatterjee, 2009).

The involvement of the anterior pituitary hormones, LH and FSH, in the control of oestrous cycle has gone beyond speculations (Dong *et al.,* 1994). In this study, there was significant decrease in the concentration of LH in stressed animals sacrificed in the oestrus phase of the cycle compared with corresponding phase in control. However, there was no significant difference in LH concentration in animals sacrificed at diestrus phase compared with control. This result is consistent with many findings in literature (Dubey and Plant, 1985; Roozendaal *et al.,* 1995; Nagatani *et al.,* 1996; Whirledge and Cidlowski, 2010) reporting the effect of different stressors and significantly elevated glucocorticoid on LH secretion. In fact, increase in glucocorticoid concentration as a result of stress has been shown to block pituitary tissue concentration of GnRH and the responsiveness of the gonadotrophs to GnRH with a resulting attenuation of LH pulse frequency (Saketos *et al.,* 1993; Smith *et al.,* 2006; Breen *et al.,* 2007). On the impact of predator-induced stress on FSH secretion, significant decrease was observed in stressed animals sacrificed at oestrus while significant increases were observed in diestrus phases of the oestrous cycle (Figure 5). Decrease in oestrus phase FSH is an indication that the post-ovulatory surge of FSH is absent (Rodriguez-Echandia *et al.,* 1998). Although this post-ovulatory surge is not dependent on GnRH secretion, it is assumed that stress-induced elevated glucocorticoid is acting centrally to depress the secretion of FSH in the oestrus phase of the cycle. However, FSH secretion of the diestrus phase of the cycle was not significantly affected. Probable mechanism for this observation may be the insignificant effect of elevated glucocorticoid on level of oestradiol secretion at this phase of the oestrous cycle.

In conclusion, PS disrupts the oestrous cycle in rat which is secondary to hormonal perturbations across the oestrous cycle. In addition, the phase at first exposure to stress has modulatory effects on the pattern of neuroendocrine response to stress. Observations made from this study show that the oestrous cycle is most vulnerable to stress when first exposed at proestrus and oestrus phases.

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