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Hormonal concentrations and reproductive performance of Holstein heifers fed *Trifolium alexandrinum* as a phytoestrogenic roughage

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ABSTRACT

Effects of phytoestrogen isoflavones in Berseem clover on hormonal balance during early pregnancy and fertility of heifers were studied. Holstein heifers ($n = 26$) were divided into two equal homogenous groups. Heifers in the first group (Clover-fed group) were fed Egyptian clover (*Trifolium alexandrinum*) as oestrogenic roughage; whereas the heifers in the second group (Silage-fed group) were fed maize silage. Concentrations of four isoflavone aglycones (genistein. Daidzein, biochanin A and formononetein) were determined in the two roughages. Treatment lasted for 20 consecutive weeks (5 months) during which blood samples were collected biweekly for determining the metabolic profile of heifers. Heifers were subjected to oestrous synchronisation using a double prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) based protocol, 14 days apart (week 10 and 12), and were artificially inseminated 12 h following detection of overt signs of oestrus. Concentrations of serum oestradiol (E_2) and progesterone (P_4) on the day of insemination (Day 0) and on Days 7, 14 and 21 post-insemination (early pregnancy) were determined and P_4 to E_2 ratio was calculated. Clover had greater total isoflavone content than maize silage. The dominant isoflavone detected in Berseem clover was biochanin A, whereas the least was formononetein. Metabolic profile was not affected ($P > 0.05$) by the type of roughage and was in the same trend in both experimental groups. The overall mean concentration of serum E_2 was greater ($P < 0.05$) in the clover-fed group than in the silage-fed group. The overall mean concentration of serum P_4 was less ($P < 0.05$) in the clover-fed group than in the silage-fed group. During the period of early pregnancy (from Day 7–21 post-insemination) the concentration of serum P_4 increased in the silage-fed group, however, no change was observed in the clover-fed group. The overall mean of P_4 to E_2 ratio was greater ($P < 0.001$) in the silage-fed group compared with that in the clover-fed group. Heifers fed clover had a lesser ($P = 0.054$) conception rate and a greater ($P = 0.062$) percentage of heifers returning to oestrus than silage-fed heifers. In conclusion, isoflavones in Berseem clover apparently disturbed hormonal balance during early pregnancy leading to a reduction in the fertility of heifers.

1. Introduction

Phytoestrogens are non-steroidal polyphenolic secondary metabolites that are naturally produced by plants. The putative effects of phytoestrogens are based on the

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structural similarity of these metabolites to the mammalian oestrogen, 17β-oestradiol (E_2), and thus potency to bind with mammalian E_2 receptors. The richest sources of phytoestrogens in animal diets are legumes, particularly soybeans and clover (Retana-Márquez et al., 2012) which have most of the phytoestrogen content in the form of isoflavones (Jefferson et al., 2012). The principal isoflavones found in clover are formononetin, daidzein, genistein and biochanin A (Blache et al., 2008; Bora and Sharma, 2011). Effects of certain legume species on animal reproduction vary widely, and range from complete to temporal infertility. Isoflavone-dependent infertility is occurring more frequently without observable symptoms and can only be detected by measuring phytoestrogens in the diet or by observation of the effects on the animal's reproductive health and efficiency (Adams, 1995). Long term consumption of isoflavones increases number of inseminations, infertility rates and early embryonic loss (Wocławek-Potocka et al., 2005; Piotrowska et al., 2006). In Egypt, *Trifolium alexandrinum*, the so-called Egyptian clover or Berseem clover is considered the primary green roughage used for animal feeding from late autumn to late spring (Hashem and Sallam, 2012). Numerous studies have recorded various reproductive disorders during the period of clover feeding in different farm animals (Kallela et al., 1984; Rachuonyo et al., 2005). There have been few studies, however, assessing the effect of phytoestrogens in Berseem clover on reproductive performance of farm animals, and the results from these studies have been inconclusive. Sharaf and Gomaa (1971) confirmed the oestrogen activity contained in Berseem clover and Hashem and Sallam (2012) suggested that Berseem clover may be one of the reasons for the occurrence of ovulation without signs of behavioural oestrus in seasonal anoestrus ewes. Shehata et al. (1982), however, reported that the concentrations of isoflavones in Berseem clover are not in great enough quantity to induce deleterious effects on reproduction or growth. The aim of the present study was to identify the isoflavone content in *T. alexandrinum* and to evaluate the reproductive performance of heifers consuming this roughage from pre-insemination to the early pregnancy period.

2. Materials and methods

This study was conducted at the Milk Production Project Facility, Faculty of Agriculture, Alexandria University, Alexandria (31°20′N, 30°E), Egypt. The procedures imposed on the animals were conducted in ways that met the Directive 2010/63/EU of the European Parliament and of the Council established on 22 September 2010 for the protection of animals used for scientific purposes.

2.1. Experimental design

Holstein heifers ($n = 26$) that were 17.28 ± 0.71 months of age, and weighing 283.30 ±11.23 kg at the time of treatment allocation were used in the present study. Heifers were equally divided into two homogenous groups, 13 heifers each according to the two roughage treatment groups. Each group was placed in an open barn with shade. The animals had free access to fresh water *ad libitum* and salt blocks. All heifers were diseases-free with a healthy appearance. The heifers in the clover-fed group were fed *T. Alexandrinum* (Berseem clover; phytoestrogenic roughage), whereas heifers in the silage-fed group were fed maize silage (non-oestrogenic roughage). Additionally, heifers were fed a legume-free concentrate mixture containing 365 g/kg maize grains; 350 g/kg cottonseed meal; 250 g/kg wheat bran; 20 g/kg limestone; 10 g/kg NaCl; and 5 g/kg mineral premix. The chemical analysis of the experimental diets is shown in Table 1. The amounts of feeds offered to the heifers in both groups were balanced to be isocaloric and isonitrogenous, considering the daily nutritional requirements (NRC, 2001). This feeding system lasted for a period of 20 consecutive weeks (five consecutive months). The schematic design of the study is depicted in Fig. 1.

Table 1
Chemical composition (g/kg) of the concentrate diet and roughage (Berseem clover or maize silage) on a dry matter (DM) basis.

Chemical composition	Concentrate	Clover	Silage
Ash	54.0	152.8	83.6
Organic matter	946.1	849.4	916.3
Ether extract	17.9	24.7	14.1
Crude protein	143.8	155.3	76.8
Neutral detergent fiber	453.6	497.8	671.7
Acid detergent fiber	281.2	324.7	379.9
Acid detergent lignin	68.5	73.5	54.2
Cellulose	212.7	251.1	325.7
Hemicellulose	172.3	173.1	298.1
Metabolisable energy (MJ/kg DM)	11.23	8.86	7.54

2.2. Oestrus and insemination management

Heifers in both groups were subjected to oestrous synchronisation by administration of a double i.m. injection of 2 ml prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Estrumate, 250 μg cloprostenol/ml, Schering-Plough Animal Health, Germany), 14 days apart (week 10 and 12). Symptoms of behavioural oestrus were monitored daily through visual observation by trained personnel every 6 h for 30 min at each observational period. Heifers were considered in oestrus based on the appearance of any of the following behavioural signs of oestrus: restlessness, sniffing/licking of vulva, frequent micturition, tail raising, bellowing, chin resting, and mounting other cows or standing to be mounted. Heifers displaying overt signs of oestrus were artificially inseminated twice, 12 and 24 h following detection of oestrus (first service). Heifers that failed to exhibit signs of oestrus after oestrous synchronisation received a third $PGF_{2\alpha}$ injection 7 days following the second $PGF_{2\alpha}$, treatment, and were inseminated in the same manner. Females that returning to oestrus after the first service were re-inseminated twice 12 h apart (second service). Frozen semen straws from the same bull were used in insemination to avoid any effect related to the male. Pregnancy was diagnosed 60 days post-insemination using rectal palpation performed by the veterinarian.