

# Effects of age, nursing, and oral IGF1 supplementation on neonatal porcine cervical development

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## Abstract

Nursing supports neonatal porcine uterine and testicular development, however, lactocrine effects on cervical development are undefined. Studies were conducted to determine the effects of i) age and the imposition of the lactocrine-null state from birth (postnatal day 0 (PND0)) by milk replacer feeding on cervical histology; ii) imposition of the lactocrine-null state for 2 days from birth on cervical cell proliferation, as reflected by proliferating cell nuclear antigen immunostaining; and iii) a single feeding of colostrum or milk replacer, administered at birth, with or without oral IGF1, on cervical cell proliferation and phosphorylated AKT (pAKT) and B-cell lymphoma 2 (BCL2) protein levels at 12 h postnatal. Cervical crypt depth and height of luminal epithelium (LE) increased with age by PND14, when both responses were reduced in replacer-fed gilts. Cell proliferation was reduced in LE at PND2, and in crypt epithelium and stroma by PND14 in replacer-fed gilts. Returning replacer-fed gilts to nursing on PND2 did not rescue the cervical phenotype by PND14. A single feeding of colostrum, but not milk replacer, was sufficient to support cervical cell proliferation at 12 h postnatal. IGF1 supplementation induced cell proliferation in replacer-fed gilts, and increased cervical pAKT and BCL2 levels in colostrum-fed gilts and replacer-fed gilts at 12 h postnatal. Results indicate that age and nursing support porcine cervical development, support is initiated at first ingestion of colostrum, IGF1 may be lactocrine-active, and identification of lactocrine-active factors can be accomplished by 12 h postnatal using this bioassay system.

*Reproduction* (2014) **148** 441–451

## Introduction

In mammals, maternal influence on neonatal development continues after birth through lactation and nursing (Peaker 2002). Nursing supports development of the neonatal porcine reproductive tract by delivering milk-borne bioactive factors (MbFs) from mother to offspring as proposed in the lactocrine hypothesis (Yan *et al.* 2006, Bartol *et al.* 2008). In the neonatal pig, lactocrine signaling is important for uterine (Miller *et al.* 2013) and testicular (Rahman *et al.* 2014) development. However, the extent to which age and nursing affect cervical histogenesis during the early postnatal period is unknown. Histologically, the adult porcine cervix is lined by columnar luminal epithelium (LE) and contains epithelial crypts that penetrate the adluminal stroma (St; Eldridge-White *et al.* 1989, Winn *et al.* 1993). Features of neonatal porcine uterine development are defined more completely with respect to both patterns of endometrial histogenesis (Bartol *et al.* 1993) and periods

of postnatal life, during which disruption of the uterine developmental program can affect the organizational trajectory and, ultimately, phenotype of the endometrium (Tarleton *et al.* 2001). For the porcine uterus, imposition of the lactocrine-null state for 48 h from birth (postnatal day 0 (PND0)), by feeding milk replacer in lieu of nursing, alters patterns of endometrial development (Miller *et al.* 2013). Effects of imposition of the lactocrine-null condition on the uterus, detected as early as PND2, were pronounced by PND14 (Miller *et al.* 2013).

Colostrum (first milk) contains bioactive factors including relaxin, a prototypical MbF (Frankshun *et al.* 2011), detected in the circulation of nursed pigs by 12 h postnatal (Yan *et al.* 2006). Insulin-like growth factor 1 (IGF1; 7.6 kDa), another member of the insulin family of hormones, is found in porcine colostrum at high concentrations (Simmen *et al.* 1990a, Donovan & Odle 1994, Donovan *et al.* 1994), which decline within the first 24–36 h of lactation during the transition from colostrum to mature milk (Klobasa *et al.* 1987,

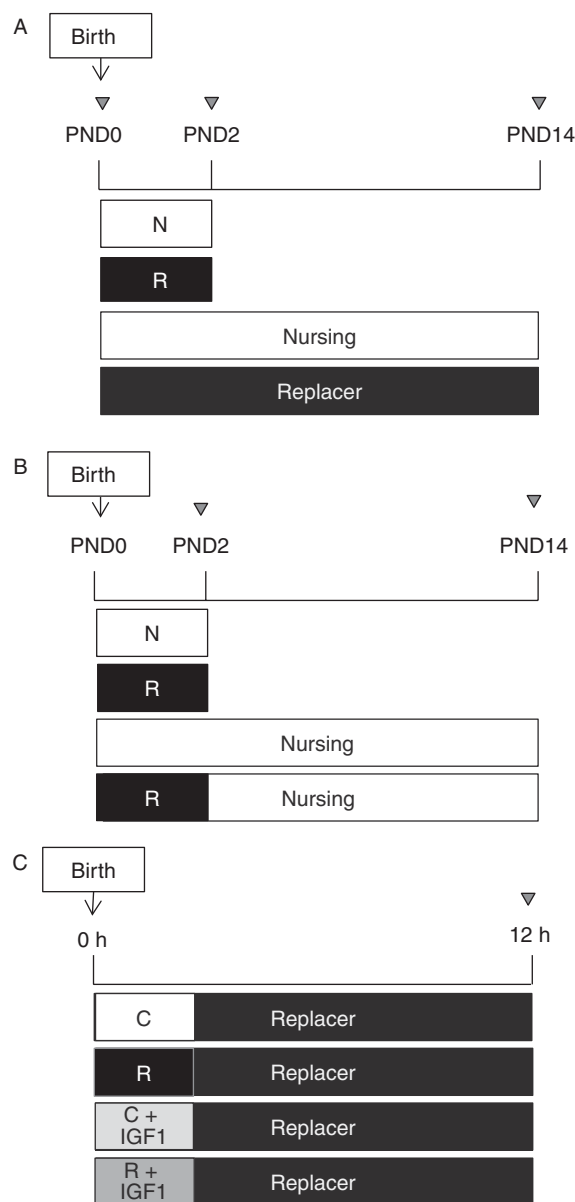
Neville *et al.* 2001, Nguyen *et al.* 2001). In the porcine neonate, a window for transmission of macromolecules, including MbFs, is open before gut closure, which is estimated to occur within 48 h of birth (Leece 1973). Orally administered IGF1 is locally bioactive in neonatal pigs, promoting intestinal crypt cell proliferation and increasing villus height (Xu *et al.* 1994, Burrin *et al.* 1996, Houle *et al.* 2000). Whether oral IGF1 affects somatic tissue development beyond the gastrointestinal (GI) tract is unknown. IGF1 signaling can act via the phosphatidylinositol-3 kinase (PI3K)/AKT pathway (Tseng *et al.* 2002, Shelton *et al.* 2004) to promote cell proliferation, differentiation, and survival (Baserga & Rubin 1993). A serine/threonine kinase (Shelton *et al.* 2004), AKT, activated when phosphorylated AKT (pAKT), increases the levels of the anti-apoptotic protein B-cell lymphoma 2 (BCL2) (Mora *et al.* 2005). Thus, broadly, pAKT and BCL2 can be used as indicators of IGF1-mediated signaling.

Three studies were conducted to evaluate the extent to which age and imposition of the lactocrine-null state from birth (PND0), as well as oral IGF1 supplementation, affect the patterns of cervical development in the neonatal gilt. The objective of study 1 was to determine the effects of age and imposition of the lactocrine-null state from birth through milk replacer feeding on cervical histology at PND2 and PND14. The objective of study 2 was to determine the effects of imposition of the lactocrine-null state (i.e. absence of nursing) for 2 days from birth on patterns of cervical cell proliferation at PND2 and PND14. Finally, study 3 was designed to determine whether a single feeding of colostrum, as compared with milk replacer, administered at birth, with or without supplemental IGF1, affects cell behaviors associated with cervical development when assessed at 12 h postnatal. Evidence of such comparatively short-term effects would support establishment of a more efficient bioassay protocol for identification of lactocrine-active factors.

## Materials and methods

### Animals

Crossbred gilts (*Sus scrofa domesticus*; Duroc, Hampshire, Yorkshire, and Landrace genetics) were obtained from the swine unit of the New Jersey Agricultural Experiment Station, Rutgers University or the Auburn University Swine Research and Education Center. At birth, gilts, born from sows at their first to fourth parity, were assigned randomly to nurse normally from their dams or to be fed porcine milk replacer (Milk Specialties Global, Carpentersville, IL, USA). Replacer-fed gilts were kept in a separate pen in the same environment and in close proximity to their dams. All pens were equipped with heat lamps. Gilts admitted to these studies had a minimum birth weight of 1.3 kg, based on evidence that postnatal development is retarded in lower body weight piglets (Rehfeldt & Kuhn 2006). Three studies were conducted as outlined in



**Figure 1** Experimental designs for (A) study 1; (B) study 2; and (C) study 3. Tissues were obtained at birth (PND0), before nursing, or gilts were allowed to either nurse (N) *ad libitum* or were fed milk replacer (R) from birth to PND2 or PND14. Oral IGF1 study 3 gilts (C) were gavage-fed a single feeding of colostrum (C) or milk replacer at birth, with or without IGF1, followed by milk replacer feeding until 12 h postnatal when tissues were collected. Cervices were obtained at time points as indicated by arrowheads;  $n=5-10$ /group.

Fig. 1. In all studies, care was taken to insure that treatments were balanced for potential effects of litter ( $n=22$  litters for the cervical histoarchitecture study (Fig. 1A);  $n=12$  litters for the cell proliferation study (Fig. 1B); and  $n=6$  litters for the IGF1 study (Fig. 1C)). Nursing groups were normalized by number of offspring and male siblings were not removed.

Study 1 was conducted to determine the effects of age and nursing from birth on the patterns of cervical histogenesis at PND2 and PND14 (Fig. 1A). In this study, the gilts were either

euthanized at birth ( $n=10$ ), before nursing, nursed *ad libitum* from birth to PND2 ( $n=7$ ) or PND14 ( $n=6$ ), or gavage-fed milk replacer (30 ml/kg body weight (BW) per 2 h) from birth to PND2 ( $n=5$ ) or PND14 ( $n=5$ ). The milk replacer dosages followed published recommendations (Houle *et al.* 2000). The cervixes were collected on PND0, PND2, or PND14.

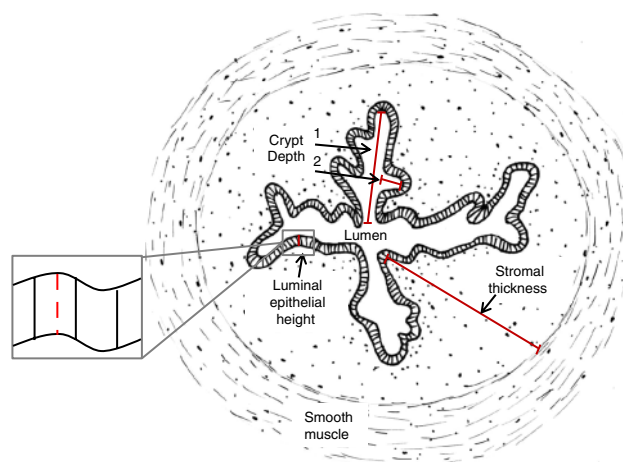
Study 2 was conducted to determine the effects of replacer feeding for 2 days from birth on cervical cell proliferation and histology at PND2 and PND14 (Fig. 1B). Here, gilts were either: i) nursed *ad libitum* from birth to PND2 ( $n=7$ ); ii) gavage-fed milk replacer from birth to PND2 ( $n=5$ ); iii) nursed *ad libitum* from birth to PND14 ( $n=5$ ); or 4) gavage-fed milk replacer for 48 h from birth and then switched to nursing until PND14 ( $n=6$ ). Tissues were collected on PND2 and PND14.

Study 3 was conducted to determine the effects of colostrum or replacer feeding at birth, with or without oral IGF1, on cervical development at 12 h postnatal (Fig. 1C). At birth, gilts ( $n=4$ –5/group) were gavage-fed a single volume (15 ml/kg BW) of either i) colostrum collected at the onset of parturition (0 h lactation); ii) colostrum supplemented with recombinant human IGF1 (1  $\mu\text{g/ml}$ ; Prepro Tech, Rocky Hill, NJ, USA); iii) milk replacer alone; or iv) milk replacer supplemented with IGF1 (1  $\mu\text{g/ml}$ ). The dose of IGF1 used in these studies was based on the concentration of IGF1 found in porcine colostrum as reported elsewhere (Simmen *et al.* 1990a). After the initial feeding, all gilts were gavage-fed milk replacer (30 ml/kg BW per 2 h) until 12 h, then they were euthanized and tissues were collected.

All procedures involving animals were reviewed and approved by relevant Institutional Animal Care and Use Committees and were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching (2010; Federation of Animal Science Societies, Champaign, IL, USA).

### Histology and histomorphometry

At the time of tissue collection, the cervixes were trimmed of excess connective tissue, fixed in Xpress Molecular Fixative (Sakura Finetek, Torrance, CA, USA) or 4% paraformaldehyde and embedded in Paraplast Plus (Sigma–Aldrich, St. Louis, MO, USA). Preliminary experiments comparing fixatives used on tissues at the same time point showed no difference on either hematoxylin and eosin or proliferating cell nuclear antigen (PCNA) staining. Cross sections of cervical tissues (6  $\mu\text{m}$ ) were affixed to Superfrost Plus slides (VWR International, LLC, Radnor, PA, USA). The tissues were deparaffinized, rehydrated, stained with Immunomaster hematoxylin (American Master Tech Scientific, Lodi, CA, USA), and counterstained with eosin (Fisher Scientific, Fair Lawn, NJ, USA). General histological observations and measurements were made in four to five nonsequential sections per gilt using an Olympus FSX100 microscope at 4.2 $\times$  and 20 $\times$  magnifications. As shown in Fig. 2, cervical crypt depth for all crypts (both deep and shallow) in each section was measured from the basal aspect of LE in the cervical crypt to the mouth of the crypt at the lumen. Porcine cervical crypts were defined as columnar epithelia forming distinct folds that protrude from the lumen. Assessment of cervical luminal epithelial height was determined by measuring



**Figure 2** Schematic of the neonatal porcine cervix (cross section) illustrating the histomorphometric protocol employed to obtain the measurements of luminal epithelial, crypt depth, and stromal thickness. Red lines show how depth of (1) deep and (2) shallow cervical crypts was measured from the basal aspect of the LE to the mouth of the crypt at the lumen. Magnified inset shows how height of the cervical LE was measured from the basal aspect to the apical surface of LE. Stromal thickness was measured from the basal border of the LE to the interface of stroma and the inner smooth muscle layer.

from the basal aspect to the apical surface of LE. A minimum of ten LE sites were chosen at random for each cervical tissue section (Ryan *et al.* 2001). Stromal thickness was measured from the basal border of the LE to the interface of St and the inner circular smooth muscle layer. Four quadrants were identified for each cervical cross-section and one measurement was made at random in each quadrant of each section.

### Immunohistochemistry

Immunostaining to detect PCNA *in situ* was carried out using a VectaStain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA). The cervical sections were subjected to antigen retrieval in boiling sodium citrate buffer (pH=6), blocked with normal horse serum at room temperature, and incubated with mouse anti-rat PCNA IgG (1:100; 0.91  $\mu\text{g/ml}$ ; Invitrogen Life Technologies) overnight at 4 °C. After incubation with biotinylated secondary antibody, endogenous peroxidase activity was blocked (3% hydrogen peroxide for 5 min at room temperature) and sections were incubated with ABC reagent and developed using 0.1% diaminobenzidine substrate (Santa Cruz Biotechnology, Dallas, TX, USA). Negative control sections were incubated with mouse isotype control IgG (1.06  $\mu\text{g/ml}$ ; Invitrogen). The images were obtained using a QImaging camera and analyzed using ImageJ Software (National Institute of Health, Bethesda, MD, USA).

### PCNA labeling index

Digital images (10 $\times$  magnification) of PCNA-stained cervical tissues were converted to 8-bit grayscale using Adobe Photoshop (CS5 Version 12.1; Adobe Systems, Inc.). The nuclei were identified as positive when staining intensity values were at or



above 30% of the highest values generated by ImageJ (Masters *et al.* 2007, Miller *et al.* 2013). For LE, crypt epithelium (CE), and St, a minimum of 1000 cells (labeled and unlabeled) were counted for each gilt. Grayscale images were converted to pseudocolor using ImageJ in order to better illustrate staining intensity. In this study, positively stained cells appear yellow–green on a black background. For each cell compartment (CE, LE, and St), a PCNA labeling index (LI), expressed as a percentage, was determined by dividing the number of PCNA-positive cells by the total number of cells counted and multiplying that value by 100 (Masters *et al.* 2007, Miller *et al.* 2013).

### Protein extraction and immunoblot analysis

Total cervical protein was extracted by homogenizing tissues (20 mg) in lysis buffer (1% Triton X-100, 10% glycerol, 150 mM Tris–HCl, 300 mM NaCl, and 1 mM MgCl<sub>2</sub>, pH=7.5). The samples were centrifuged (12 000 *g*/4 °C/10 min) and the protein supernatant was removed and stored at –80 °C. Protein concentrations were measured using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Cervical proteins (20 µg) were resolved on 12% total monomer, Bis–Tris–HCl-buffered polyacrylamide gels (Invitrogen) under reducing conditions, followed by transfer onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked with 10% nonfat milk powder (NFMP) in Tris–buffered saline containing Tween (TBST; 25 mM Tris (pH=7.5), 0.14 mM NaCl, 3 mM KCl, and 0.05% Tween 20) for 1 h at room temperature. The membranes were incubated with rabbit anti-mouse pAKT (1:1000; 60 kDa; Cell Signaling, Beverly, MA, USA) in TBST–5% NFMP overnight at 4 °C. The membranes were then washed with TBST and incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:10 000; Invitrogen) for 1 h at room temperature. After washing in TBST, the membrane-bound antibodies were detected by ECL (Perkin Elmer Life Sciences, Waltham, MA, USA) on film, as per manufacturer recommendations. The membranes were washed with stripping buffer (Restore Western Blotting Stripping Buffer, Thermo Scientific, Waltham, MA, USA) to remove antibodies. To ensure that all antibodies were removed after stripping, ECL was added and membranes were developed using film to confirm that no signal was present. The membranes were then incubated with rabbit anti-mouse AKT (1:1000; 60 kDa; EMD Millipore, Burlington, MA, USA) overnight at 4 °C, washed with TBST, and incubated with goat anti-mouse IgG (1:10 000; Invitrogen) for 1 h at room temperature. Signal was detected using ECL and film. Lastly, the membranes were probed with rabbit anti-human BCL2 (1:1000; 26 kDa; sc-492, Santa Cruz Biotechnology) overnight at 4 °C, washed with TBST, and incubated with goat anti-rabbit IgG (1:10 000; Invitrogen) for 1 h at room temperature. The pAKT controls were lysates (10 µg) from bovine mammary epithelial cells (MAC-T) incubated with (+ control) or without (– control) IGF1 (Fleming *et al.* 2006). The BCL2-positive control lysate was human BCL2-transfected 293T cells (10 µg; sc-176463; Santa Cruz Biotechnology). Protein loading was monitored using total AKT. For AKT, pAKT, and BCL2, antibody specificity was evaluated by omission of primary antibodies in the immunoblot analysis. Incubation with secondary antibody alone showed no evidence of cross reactivity with cervical proteins.

Chemiluminescence signals were quantified densitometrically from the film using Scion Image for Windows (Scion Corp., Frederick, MD, USA). All data were corrected for background signals and expressed as target protein to total AKT ratio.

### Statistical analyses

Quantitative data were subjected to the analyses of variance using GLM procedures (SAS 2013, Cary, NC, USA) and are presented as least squares means (LSM) ± S.E.M. For studies 1 and 2 analyses considered variation due to the main effects of treatment (nursing vs replacer), age, and cell compartment (epithelium vs St), as well as interactions as appropriate. For study 3, analyses considered variation due to the main effects of a single feeding of colostrum or milk replacer with or without oral IGF1 (colostrum vs replacer feeding; colostrum alone vs colostrum + IGF1; and replacer alone vs replacer + IGF1).

## Results

### Study 1: effects of age and nursing on cervical histoarchitecture

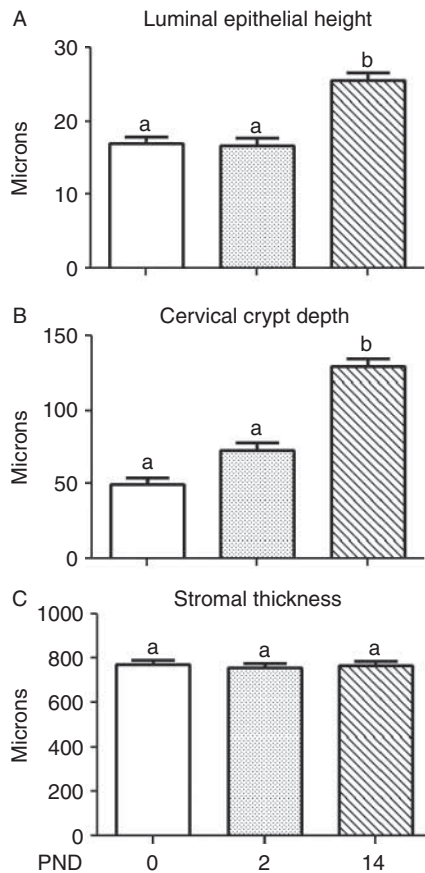
The effects of neonatal age on cervical luminal epithelial height, crypt depth, and stromal thickness are shown in Fig. 3. Both luminal epithelial height (Fig. 3A) and crypt depth (Fig. 3B) were similar on PND0 and PND2, and increased ( $P < 0.001$ ) by PND14. Stromal thickness was not affected by age.

Figure 4 shows the effects of neonatal age and milk replacer feeding on cervical histoarchitecture. Overall, both luminal epithelial height and crypt depth increased ( $P < 0.001$ ) from PND2 to PND14. Imposition of the lactocrine-null state from birth by replacer feeding reduced ( $P < 0.001$ ) luminal epithelial height and crypt depth by PND14, when treatment effects were most pronounced (treatment × day,  $P < 0.001$ ). In contrast, stromal thickness was unaffected by either age or treatment.

Cervical histology on PND0 and PND14 in nursed and replacer-fed gilts is shown in Fig. 5. Cervical crypt development advanced from PND0 to PND14 and was most pronounced in nursed gilts in which, compared with replacer-fed gilts, more extensive branching of cervical crypts was observed by PND14. Cervices from replacer-fed gilts obtained on PND14 were histologically similar to the tissues obtained on PND0.

### Study 2: effects of age and nursing on cervical cell proliferation

Pseudocolored images showing the effects of nursing for 2 days from birth on PCNA immunostaining patterns and histograms depicting PCNA LI data for cervical tissues obtained on PND2 and PND14 are shown in Fig. 6. Signal indicative of PCNA labeling above background was observed in all cell compartments. On PND2, cervical histology of nursed gilts (Fig. 6A) was similar to that of replacer-fed gilts (Fig. 6B). However, the



**Figure 3** Effects of age on neonatal porcine cervical (A) luminal epithelial height, (B) crypt depth, and (C) stromal thickness. Data are expressed as  $LSM \pm S.E.M.$ . Different letters denote differences ( $P < 0.001$ ).

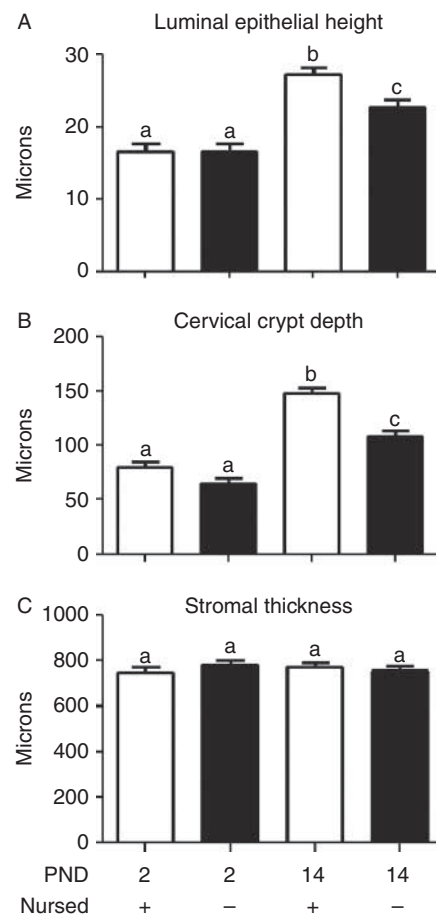
imposition of the lactocrine-null state for 2 days from birth reduced ( $P < 0.05$ ) PCNA LI compared with nursed gilts (Fig. 6C) by PND2. Overall, in nursed and replacer-fed gilts, PCNA LI was lower ( $P < 0.01$ ) in epithelium (LE and CE) when compared with St on PND2. This effect was most pronounced for LE (treatment  $\times$  cell compartment,  $P < 0.05$ ).

Generally, in comparison with gilts nursed for 2 weeks from birth, cervical development on PND14 was retarded in replacer-fed gilts that returned to nursing on PND2 (Fig. 6D vs E). Overall, PCNA LI was lower ( $P < 0.001$ ) in epithelium than in St (Fig. 6F), and higher ( $P < 0.05$ ) in nursed as compared with replacer-fed gilts. Reduced PCNA LI associated with the imposition of the lactocrine-null condition for 2 days from birth was most pronounced in CE and St (treatment  $\times$  cell compartment,  $P < 0.05$ ).

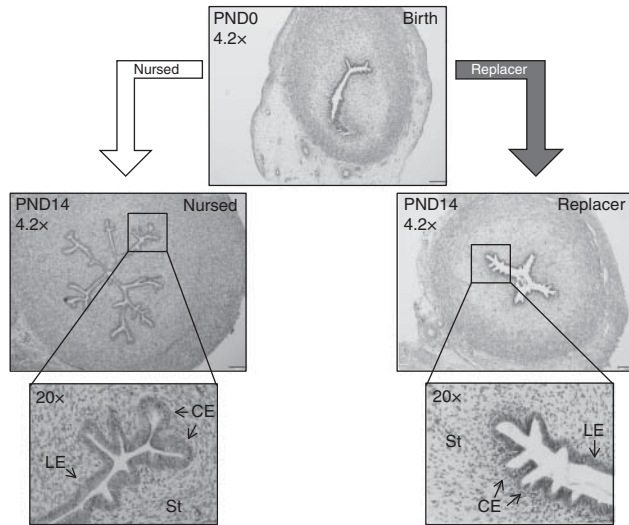
### Study 3: effects of a single feeding of colostrum or milk replacer, with or without oral IGF1, on cervical cell proliferation at 12 h postnatal

Images showing the effects of a single feeding of colostrum or milk replacer at birth, with or without IGF1, on cervical PCNA immunostaining patterns, and

PCNA LI at 12 h postnatal are shown in Fig. 7. In comparison with gilts given a single feeding of colostrum at birth, replacer feeding alone was associated with a marked reduction in PCNA immunostaining at 12 h (Fig. 7A vs B). Colostrum-fed gilts showed similar PCNA immunostaining patterns with or without oral IGF1 (Fig. 7A vs C). In contrast, IGF1 supplementation for replacer-fed gilts resulted in cervical immunostaining patterns similar to those observed for colostrum-fed gilts (Fig. 7D vs A and C). These observations were reflected by data for PCNA LI (Fig. 7E, F and G). For cervical LE ( $P < 0.01$ ), CE ( $P < 0.001$ ), and St ( $P < 0.05$ ), PCNA LI at 12 h postnatal was reduced in replacer-fed gilts compared with colostrum-fed gilts. The colostrum-fed gilts supplemented with IGF1 showed similar PCNA LIs compared with those observed for gilts fed colostrum alone in all cervical compartments. However, when replacer-fed gilts were supplemented with IGF1, PCNA LI for LE, CE, and St were increased in comparison with gilts fed replacer alone ( $P < 0.01$ ), and were comparable with those observed for colostrum-fed gilts, with or without supplemental IGF1.



**Figure 4** Effects of nursing (+) or milk replacer feeding (-) and neonatal age on (A) luminal epithelial height, (B) crypt depth, and (C) stromal thickness. Data are expressed as  $LSM \pm S.E.M.$ . Different letters denote differences ( $P < 0.01$ ).



**Figure 5** Cervical histoarchitecture affected by age and nursing. Photomicrographs of porcine cervical tissues obtained at birth (PND0; top) and from nursed (left) as compared with replacer-fed (right) gilts on PND14. Low-power images (4.2 $\times$ ; bar = 153  $\mu$ m) and high-power detailed (20 $\times$ ; bar = 32  $\mu$ m) images illustrate the effects of both age (PND0 vs PND14) and imposition of the lactocrine-null condition from birth by replacer feeding on cervical histology. Crypt epithelium (CE), LE, and stroma (St) are indicated in 20 $\times$  images.

### Study 3: effects of a single feeding of colostrum or milk replacer, with or without oral IGF1, on cervical BCL2 and pAKT proteins at 12 h postnatal

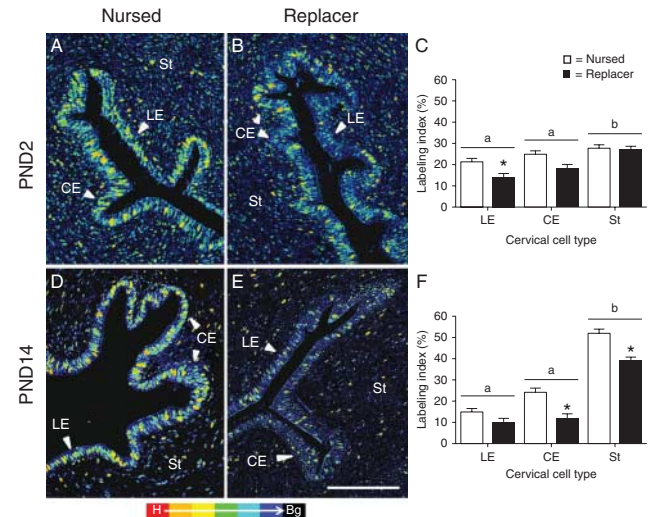
A representative immunoblot image showing BCL2, pAKT, and total AKT proteins at 12 h postnatal in cervical protein lysates from colostrum-fed gilts or replacer-fed gilts, with or without oral IGF1, is shown in Fig. 8A. Immunoreactive BCL2 (26 kDa), pAKT (60 kDa), and AKT (60 kDa) proteins were detected in all cervical tissues. Colostrum-fed gilts and replacer-fed gilts showed comparable expression of BCL2 protein. However, oral supplementation of IGF1 in colostrum-fed gilts increased cervical BCL2 ( $P < 0.05$ ) levels when compared with control gilts fed colostrum alone. Likewise, replacer-fed gilts supplemented with IGF1 had higher cervical BCL2 ( $P < 0.05$ ) protein levels when compared with gilts fed replacer alone (Fig. 8B). Cervical pAKT protein levels were similar in gilts fed colostrum or replacer alone. Cervical pAKT levels increased ( $P < 0.001$ ) in colostrum-fed gilts supplemented with IGF1 when compared with gilts fed colostrum alone. Similarly, IGF1-supplemented, replacer-fed gilts had increased ( $P < 0.001$ ) pAKT protein levels when compared with gilts fed only replacer (Fig. 8C).

## Discussion

Results confirm and extend previous findings for the porcine uterus (Miller *et al.* 2013) and testes (Rahman *et al.* 2014), by showing the effects of age and nursing on

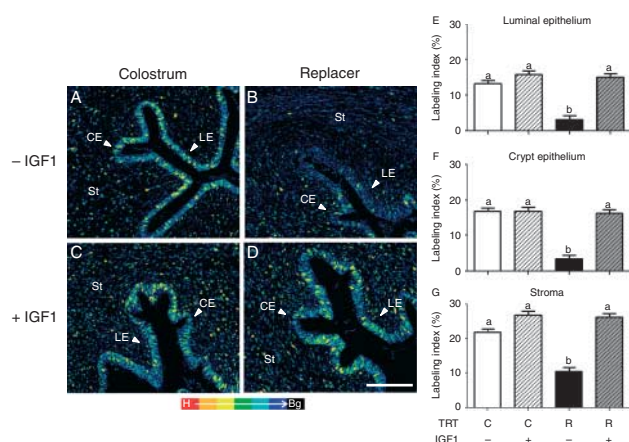
cervical development that are evident on or before PND14. New observations were presented regarding porcine cervical histogenesis that illustrate the effects of imposition of the lactocrine-null state for 2 days from birth on cellular and molecular aspects of cervical development in the neonate. Furthermore, results indicate that a single feeding of colostrum at birth affects events associated with cervical development that are detectable by 12 h postnatal, and that such effects can be mimicked by oral IGF1 supplementation in the neonatal pig.

In nursed pigs, events associated with cervical histogenesis reported in this study include age-related increases in crypt depth, luminal epithelial height, and branching of crypts into the cervical St. The results indicate that, as observed for the uterus (Miller *et al.* 2013), cervical histology does not change appreciably between birth and PND2, and that cervical crypt depth and luminal epithelial height increase with age by PND14. However, as observed for endometrial gland development in the neonatal pig (Miller *et al.* 2013), cervical crypt development was retarded on PND14 in lactocrine-null gilts. In the adult pig, cervical crypts form interdigitations important for copulation, insemination, and fertility (Eldridge-White *et al.* 1989, Winn *et al.* 1993). Whether alterations in cervical development reported in this study for replacer-fed gilts affect reproductive performance later in life has yet to be determined. However, a relationship between the



**Figure 6** Effects of neonatal age and nursing on cervical PCNA immunostaining and PCNA labeling indices (LIs). Pseudocolored images illustrate PCNA labeling patterns in LE, crypt epithelium (CE), and stroma (St) for cervical tissues obtained on PND2 (A and B) and PND14 (D and E) from nursed (A and D) and replacer-fed (B and E) gilts. Positively, immunostained cells appear yellow–green on a black background; scale bar indicates 100  $\mu$ m. Associated data for PCNA LI by cervical cell compartment are shown for PND2 (C) and PND14 (F). White bars denote nursed and black bars denote replacer-fed groups. Data are presented as LSM  $\pm$  s.e.m. Horizontal lines with different letters denote differences between cell compartments at PND2 ( $P < 0.01$ ) and PND14 ( $P < 0.001$ ). Asterisks denote cell compartment-specific treatment effects (nursed vs replacer) at PND2 and PND14 ( $P < 0.05$ ).





**Figure 7** Effects of a single feeding of colostrum (C) or milk replacer (R), with (+) or without (-) oral IGF1, on cervical PCNA immunostaining patterns (A, B, C and D) and PCNA LI (E, F and G) at 12 h postnatal. Pseudocolored images illustrate PCNA labeling patterns in LE, CE, and St. Positively stained cells appear yellow-green on a black background; scale bar indicates 100  $\mu$ m. For histograms, data are presented as LSM  $\pm$  S.E.M. and different letters denote differences (LE,  $P < 0.01$ ; CE,  $P < 0.001$ ; and St,  $P < 0.05$ ).

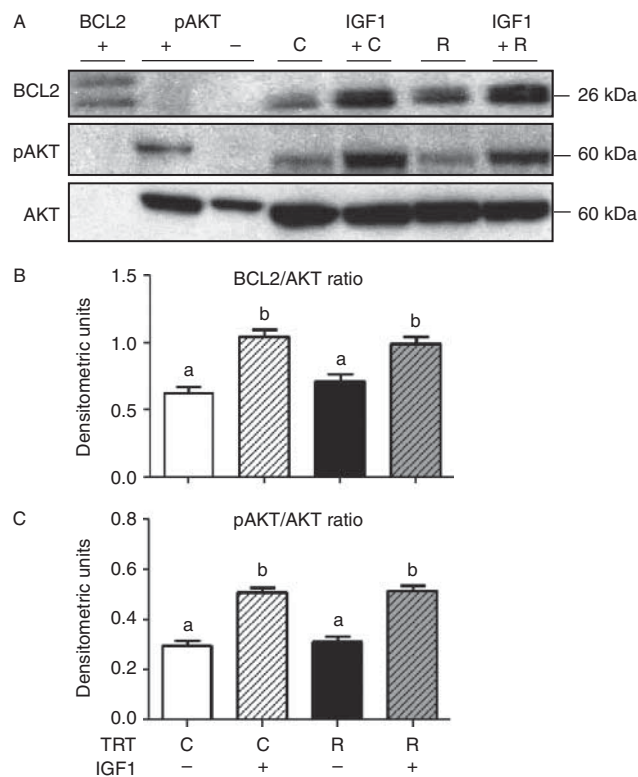
amount of colostrum consumed by gilts on PND0 and their lifetime fecundity was documented (Bartol *et al.* 2013). In a retrospective study involving 381 sows, low serum immunoglobulin immunocrit values on their day of birth (Vallet *et al.* 2013), indicating minimal colostrum consumption, were associated with reduced litter size over four parities. Taken together, studies emphasize the importance of colostrum ingestion shortly after birth for the support of female reproductive tract (FRT) development. Mechanisms through which lactocrine programming of FRT development may affect reproductive capacity remain to be defined.

Targeted disruption of FRT development during organizationally critical periods of postnatal life can have lasting effects on the form and function of adult reproductive tissues (Crain *et al.* 2008, McLachlan *et al.* 2012). In the hamster, neonatal exposure to diethylstilbesterol increased cervical diameter and altered LE histology, reflected by increased luminal epithelial height and cornification, in a manner that persisted into adulthood (Hendry *et al.* 2004). In sheep (Bartol *et al.* 1999, Gray *et al.* 2001) and mice (Cooke *et al.* 2012, Filant *et al.* 2013), strategic neonatal exposure to progestins inhibited the development of uterine glands and suppressed fertility in adults. Such lasting effects are likely to reflect the disruption of postnatal organizational events that determine cell fate and, by consequence, morphogenetic processes that dictate tissue structure and function (Bartol & Bagnell 2012).

Although cervical histology at PND2 was similar in nursed and replacer-fed gilts, reduced proliferation of LE was observed in replacer-fed gilts as reflected by PCNA LI. Treatment effects on the patterns of cervical LE

proliferation were similar to those described for the PND2 porcine endometrium (Miller *et al.* 2013). Consistently, as observed for the neonatal porcine uterus (Miller *et al.* 2013), imposition of the lactocrine-null state from birth reduced PCNA LI in CE and St, but not in LE, on PND14. Collectively, results are consistent with the idea that lactocrine support is important for both cervical and endometrial development in the neonatal pig, and that mitogenic factors in colostrum support development (Donovan & Odle 1994, Blum & Baumrucker 2008).

Returning replacer-fed gilts to nursing on PND2 did not rescue the cervical phenotype by PND14. Again, results obtained for the cervix agreed with the observations reported for the developing porcine endometrium (Miller *et al.* 2013). Factors to be considered in explanation of these observations include potential interactions of GI tract maturation with parallel changes in the composition of colostrum that are associated with its transition to mature milk. In the pig, gut closure occurs within 24–48 h of birth (Leece 1973). This event



**Figure 8** Effects of a single feeding of colostrum (C) or milk replacer (R), with (+) or without (-) oral IGF1, on cervical BCL2 and phosphorylated (p)AKT proteins at 12 h postnatal. Representative immunoblot (A) images show positive (+; BCL2 and pAKT) and negative (-; pAKT) control samples as described in 'Materials and methods' section. Densitometric data for the relative expression of BCL2 (B) and pAKT (C) were normalized to total AKT and expressed as LSM  $\pm$  S.E.M. Different letters indicate differences (BCL2,  $P < 0.05$  and pAKT,  $P < 0.001$ ).

prevents further transfer of macromolecules, including potential MbFs, across the GI tract and into the neonatal circulation. Milk-replacer feeding can shorten the period from birth to gut closure to as early as 12 h postnatal (Jensen *et al.* 2001), thereby narrowing the window for MbF transfer. In parallel with such changes in GI tract function, changes in colostrum composition also occur within 48 h of parturition in the pig (Klobasa *et al.* 1987, Ogawa *et al.* 2014). Such changes may alter the nature and array of available lactocrine-active factors with potential to be transferred to the neonate by PND2. This is when replacer-fed gilts were returned to nursing in study 2. The observation that mammatrope development was delayed throughout the prepubertal period when neonatal rats, deprived of lactation day 1 colostrum, were cross-fostered to dams on lactation day 7 supports this idea (Nusser & Frawley 1997). Both changes in neonatal gut closure dynamics and colostrum composition over time may define the critical window for lactocrine signaling.

Results of study 3, in which a single feeding of either colostrum or milk replacer was administered via orogastric gavage, confirm data reported in studies 1 and 2, indicating that colostrum consumption immediately after birth supports cervical cell proliferation. Furthermore, these data establish that such effects are detectable by 12 h postnatal. These observations provide the basis for the development of a 12 h bioassay protocol for evaluation of potential lactocrine-active factors. Data also indicate that IGF1 is orally active in the porcine neonate as reflected by the effects on cervical PCNA LI, as well as patterns of cervical BCL2 and pAKT expression.

A candidate MbF, IGF1, is present in the colostrum and milk of multiple species (Francis *et al.* 1988, Campbell & Baumrucker 1989, Nagashima *et al.* 1990, Prosser *et al.* 1990, Donovan *et al.* 1991*a,b*), including the pig (Simmen *et al.* 1988). Similar to milk-borne relaxin (Frankshun *et al.* 2012), IGF1 concentrations in porcine milk are highest during the first few days of lactation and decline during the transition of colostrum into milk (Simmen *et al.* 1990*b*, Donovan *et al.* 1994). Milk-borne IGFs are present in free and bound states throughout lactation (Donovan *et al.* 1994, Xu & Wang 1996). IGF-binding proteins (IGFBPs), present in porcine milk (Simmen *et al.* 1988, Donovan *et al.* 1994), function as transport molecules, increase the half-life of circulating IGF1 (Walton *et al.* 1989), and can modulate activity of IGF (Baxter 1988, McCusker *et al.* 1991).

Whether IGF1 administered orally can enter the neonatal porcine circulation remains unclear. Serum IGF1 concentrations did not differ between replacer-fed pigs with or without supplemental IGF1 for 4 days from birth when evaluated at 24, 48, 72, or 96 h postnatal (Burrin *et al.* 1996). Similarly, porcine serum IGF1 concentrations assessed on PND14 were unaffected by oral supplementation of IGF1 delivered in milk replacer (Houle *et al.* 2000). Nevertheless, both studies reported

proliferative effects in the GI tract for IGF1-supplemented pigs (Burrin *et al.* 1996, Houle *et al.* 2000). By contrast, serum IGF1 concentrations in neonatal rats fed formula supplemented with IGF1 for 3 days were higher than those found in rats fed formula devoid of IGF1 (Philipps *et al.* 1997). In addition, when fed orally to pigs at birth or on PND3, <sup>125</sup>I-IGF1, administered in colostrum, was detected in peripheral plasma within 1 h after administration, and was found in the stomach, small intestine, kidney, and other somatic tissues by 4 h post-administration (Xu & Wang 1996). These results indicated that orally administered IGF1 can and does enter the neonatal circulation. Consistently, in a comparison with neonatal pigs that were either nursed or replacer-fed (Dauncey *et al.* 1994), nursed pigs displayed higher plasma IGF1 concentrations and increased growth rate. In that study, hepatic IGF1 levels were similar in nursed and replacer-fed pigs, suggesting that differences in plasma IGF1 concentrations were due to high levels of milk-borne IGF1 (Dauncey *et al.* 1994). Currently, the preponderance of data supports the idea that IGF1 acquired orally can enter the neonatal circulation. To the extent that this is the case, effects of orally administered IGF1 reported here could reflect direct actions of this peptide growth factor at the level of the cervix.

Oral IGF1 can stimulate the growth of neonatal porcine GI tissues. In replacer-fed pigs, oral IGF1 promoted cell proliferation in intestinal crypts (Xu *et al.* 1994), increased jejunal and ileal villus height by PND4 (Burrin *et al.* 1996), and both jejunal villus height and mucosal DNA content and synthesis by PND14 (Houle *et al.* 2000). The present results indicate that the porcine cervix is also a target for oral IGF1. The extent to which these effects reflect direct actions of orally administered IGF1 at the level of the cervix or an alternate indirect mechanism (Baumrucker & Blum 1993) is not known.

Supplementation of colostrum with IGF1 did not affect cervical cell proliferation. However, in replacer-fed gilts oral IGF1 administration increased cervical cell proliferation to levels equivalent to those observed for colostrum-fed gilts at 12 h postnatal. One explanation for these effects is that agents in colostrum that are absent in milk replacer, including IGFBPs (Donovan *et al.* 1994), caseins, and/or lactoferrin (Xian *et al.* 1995), may bind IGF1 and impede its uptake into the neonatal circulation, thereby reducing IGF1 bioavailability. The most abundant binding protein in peripheral circulation, IGFBP3 can bind substantial amounts of circulating IGF1, thereby increasing its half-life (Baxter 1991). Also present at high concentrations in the circulation of young pigs (Dauncey *et al.* 1993), IGFBP2, together with IGFBP3, could further affect the bioavailability of IGF1. To the extent that IGFBP profiles differ between colostrum-fed gilts and replacer-fed gilts, this could explain, in part, the proliferative responses of cervical



cells seen in IGF1-supplemented, replacer-fed gilts, in which IGF1 bioavailability could be greater.

Cervical pAKT and BCL2 responses indicated that orally administered IGF1 was active in colostrum-fed gilts and replacer-fed gilts. IGF1 stimulates cell proliferation and promotes cell differentiation and survival through interactions with the IGF1 receptor and subsequent activation of signaling cascades involving the PI3K/AKT pathway (Woodgett 2005). A serine/threonine kinase, AKT plays a role in inhibition of apoptosis by phosphorylating and inactivating several proteins involved in the apoptotic cascade (Dudek *et al.* 1997, Mora *et al.* 2005). Activated pAKT can increase the levels of anti-apoptotic proteins including BCL2 (Mora *et al.* 2005).

The fact that cervical pAKT and BCL2 expression levels were similar at 12 h postnatal in gilts given a single feeding of either colostrum or milk replacer at birth indicates that cervical expression of these molecules is not acutely lactocrine sensitive. In contrast to results obtained for cervical cell proliferation, oral IGF1 supplementation increased cervical pAKT and BCL2 expression regardless of the medium in which it was delivered. An explanation for these observations may be that pAKT and BCL2 responses are more sensitive to induction by IGF1. Reduced IGF1 bioavailability in colostrum (Simmen *et al.* 1988, Xian *et al.* 1995) could also contribute to differing results, as indicated above. Furthermore, the capacity of endogenous IGF1 to bind additional, exogenous IGF1 can be limited by the degree of binding protein saturation (Umezawa *et al.* 1991). The extent to which agents in colostrum affect the bioavailability of IGF1 or other potentially lactocrine-active MbFs will be important to determine.

Taken together, the results presented in this study establish that, as reported for the neonatal porcine uterus (Miller *et al.* 2013) and testis (Rahman *et al.* 2014), as well as for the murine hippocampus (Liu *et al.* 2014), lactocrine support is necessary for normal development of the neonatal porcine cervix. Disruption of cervical development by transient imposition of the lactocrine-null state for 2 days from birth altered the cervical developmental program in a manner that, as assessed on PND14, could not be reversed by returning gilts to nursing in synchrony with their littermates. Evidence that effects of a single feeding of colostrum at birth can be detected in developing porcine cervical tissues by 12 h postnatal indicates that lactocrine regulation of postnatal events associated with cervical and, by extension, FRT development is initiated at first ingestion of colostrum. This observation provides the basis for the development of a more efficient bioassay for the identification of lactocrine-active MbFs. Present results also implicate IGF1, biologically active in the piglet, as a potential lactocrine-active factor affecting FRT development.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Funding

This work was supported by USDA-NIFA NRI grants 2007-35203-18098 and 2013-67016-20523 and NSF-EPS-1158862.

## Acknowledgements

The authors thank Laura Comerford, Felicia Kleiman, and Rebecca Potosky in the Rutgers Animal Care Program, Auburn Animal Care staff, Camp David, and the Rutgers undergraduate Sow Watch Club for their contributions to these studies.

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Received 19 May 2014

First decision 8 July 2014

Revised manuscript received 24 July 2014

Accepted 29 July 2014