Central and peripheral administration of kisspeptin activates gonadotropin but not somatotropin secretion in prepubertal gilts

Clay A Lents, Neely L Heidorn, C Richard Barb¹ and J Joe Ford²

Department of Animal and Dairy Science, College of Agricultural and Environmental Sciences, 316 Edgar L Rhodes Center for Animal and Dairy Science, The University of Georgia, 425 River Road, Athens, Georgia 30602-2771, USA, ¹Richard B Russell Agricultural Research Center, USDA-ARS, Athens, Georgia 30604-5667, USA and ²US Meat Animal Research Center, USDA-ARS, Clay Center, Nebraska 68933, USA

Correspondence should be addressed to C A Lents; Email: clents@uga.edu

Abstract

It is well established that kisspeptin signaling is necessary for the onset of puberty in laboratory animals. However, the role that kisspeptin may have in regulating puberty in large domestic animals is unknown. We tested the hypothesis that either central or peripheral infusion of kisspeptin would stimulate gonadotropin and GH secretion in prepubertal gilts. In experiment 1, prepubertal gilts were fitted with i.c.v. cannula and indwelling jugular catheters. Animals were randomly assigned to receive 0, 10, or 100 µg kisspeptin in saline. In experiment 2, prepubertal gilts, fitted with indwelling jugular catheters, randomly received 0, 1, 2.5, or 5 mg kisspeptin in saline intravenously. Serial blood samples were collected every 15 min for 3 h before and 5 h after infusions, and serum concentrations of LH, FSH, and GH were determined. Mean concentrations of LH and FSH remained at basal levels for control animals but were increased ($P < 0.001$) for animals receiving i.c.v. infusion of kisspeptin. Area under the LH and FSH curves following i.c.v. infusion of kisspeptin increased ($P < 0.001$) in a dose-dependent manner. Concentrations of GH were unaffected by i.c.v. treatment. Peripheral administration of kisspeptin increased ($P < 0.05$) serum concentrations of LH but not FSH or GH. Thus, kisspeptin can activate gonadotropic but not somatotropic hormone secretion in prepubertal gilts. The present data support the concept that kisspeptin plays a role in the mechanism involved in initiating puberty in swine.

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Introduction

Gonadotropin-releasing hormone (GnRH) is secreted from hypothalamic neurons and is transported through the hypophyseal portal veins to the anterior pituitary gland where it stimulates synthesis and secretion of luteinizing hormone and follicle-stimulating hormone (LH and FSH; Schally *et al.* 1971). These pituitary gonadotropins are essential for normal gonadal development and function (Brinkley 1981, Desjardins 1981, Clarke *et al.* 1983, Gharib *et al.* 1990). In mammals, the components of the hypothalamic–pituitary–gonadal (HPG) axis are functional prior to the normal onset of puberty (Lutz *et al.* 1984, Urbanski & Ojeda 1987), which is thought to be brought about by central and peripheral changes that lead to increased activity of the GnRH pulse generator (Pelletier *et al.* 1981, Foster *et al.* 1985, Plant *et al.* 1989). However, the mechanisms within the brain that regulate the proper temporal release of GnRH from hypothalamic neurons to initiate puberty remain largely unknown.

In general, increased excitatory signals and reduced inhibitory signals to the GnRH neuronal network prompt

the onset of puberty (Ojeda & Urbanski 1994). With regard to the former, kisspeptins have been implicated as potent stimulators of the gonadotropic axis (Gottsch *et al.* 2004, Dhillon *et al.* 2005). Structurally related peptides, kisspeptins, are products of the *KISS1* gene (Kotani *et al.* 2001, Ohtaki *et al.* 2001). Synthesized as a pre-prohormone, it is cleaved to liberate a 54 amino acid peptide that can be proteolytically processed (Takino *et al.* 2003) to shorter variants; all of which share the same amidated C terminus and retain full biological activity (Kotani *et al.* 2001, Ohtaki *et al.* 2001). Kisspeptin, acting through its cognate G-protein coupled receptor GPR54/KISS1R (Kotani *et al.* 2001), is thought to be an important determinant in the onset of puberty (Seminara & Kaiser 2005). Indeed, *Gpr54/Kiss1r* knockout mice failed to initiate puberty (Funes *et al.* 2003, Seminara *et al.* 2003) and naturally occurring mutations in *GPR54/KISS1R* result in idiopathic hypogonadotropic hypogonadism in humans (de Roux *et al.* 2003, Seminara *et al.* 2003, Semple *et al.* 2005). Expression of the *KISS1* and *GPR54/KISS1R* genes is both hormonally and developmentally regulated in the rodent (Navarro *et al.* 2004a, 2004b). Kisspeptin stimulates LH secretion (Matsui *et al.* 2004,

Thompson *et al.* 2004, Navarro *et al.* 2005b) in a GnRH-dependent manner (Gottsch *et al.* 2004, Shahab *et al.* 2005, Arreguin-Arevalo *et al.* 2007). It has been demonstrated that kisspeptin acts directly on GnRH neurons to stimulate GnRH and gonadotropin secretion (Thompson *et al.* 2004, Irwig *et al.* 2005, Messenger *et al.* 2005). This has culminated in the establishment of a role for kisspeptin in the initiation of puberty in the rodent and primate (Shahab *et al.* 2005, Castellano *et al.* 2006). Data are available with regard to the effects of kisspeptin on LH secretion in the adult ewe (Messenger *et al.* 2005, Arreguin-Arevalo *et al.* 2007, Caraty *et al.* 2007). However, the possible role of kisspeptin in mediating the onset of puberty in large domestic species, in particular the pig, has yet to be determined.

In the present study, we hypothesize that kisspeptin is an important regulator of gonadotropin secretion in the pig. To test this hypothesis, we administered kisspeptin either centrally or peripherally to prepubertal gilts and measured changes in gonadotropin secretion. Because the occurrence of puberty in the pig is directed by mechanisms in which the HPG axis regulates both gonadal function and growth (Barb *et al.* 1999), we tested the hypothesis that kisspeptin would modulate growth hormone (GH) secretion in the prepubertal gilt.

Results

Experiment 1

A treatment-time interaction ($P<0.001$) was detected for serum concentrations of LH. Prior to the i.c.v. infusion, serum concentrations of LH were similar for all animals (0.25 ± 0.03 ng/ml). Following i.c.v. treatment, LH concentrations remained at basal levels for the control animals but were increased ($P<0.001$) for animals receiving either 10 or 100 μ g kisspeptin (Figs 1 and 2). Mean concentration, peak concentration, and area under the curve (AUC) of serum LH were greater ($P<0.001$) in period 2, following i.c.v. infusion, for kisspeptin-treated animals compared with saline-treated controls (Fig. 2). Mean concentration and AUC of serum LH in period 2 were greater ($P<0.01$) for animals treated with the 100 μ g dose of kisspeptin compared with the 10 μ g dose (Fig. 2), but peak concentrations of serum LH in period 2 were similar (Fig. 2). All animals responded to a single i.v. bolus infusion of GnRH in period 3 with elevated serum LH (Fig. 1). Mean concentration and peak concentration of serum LH in period 3 were greater ($P<0.05$) for control animals compared with kisspeptin-treated animals (Fig. 2). Animals treated with the 10 μ g dose of kisspeptin had decreased ($P<0.05$) AUC

Figure 1 Serum concentrations of LH and FSH (means $\pm$ S.E.M.) for prepubertal gilts receiving i.c.v. infusion of saline or either 10 or 100 μ g kisspeptin (Kiss). Period 1 is the 3 h prior to i.c.v. treatment; period 2 is the 3 h after i.c.v. treatment; period 3 is the 90 min following an i.v. injection of 100 μ g GnRH. A treatment-time interaction was detected for LH ($P<0.001$) and FSH ($P<0.001$). *Times at which effects of treatment were different from saline-treated control animals ($P<0.05$). #Times at which effects of treatment tended to be different from saline-treated control animals ($P<0.10$).

Figure 2 Mean LH, peak LH, and area under the curve (AUC) of serum LH for prepubertal gilts receiving i.c.v. infusion of saline or either 10 or 100 µg kisspeptin (Kiss). Data in period 1 are from the 3 h prior to i.c.v. treatment; data in period 2 are from the 3 h after i.c.v. treatment; and data in period 3 are from the 90 min following an i.v. infusion of 100 µg GnRH. Data are presented as mean $\pm$ S.E.M. ^{a,b}Means within a period differ between treatments ($P < 0.05$).

following GnRH when compared with control animals treated with GnRH (Fig. 2). Area under the LH curve following GnRH was similar for control animals and those treated with the 100 µg dose of kisspeptin (Fig. 2), since serum concentrations of LH in the kisspeptin-treated animals were still above baseline at the beginning of period 3 (Fig. 1).

Similar to LH, there was a treatment–time interaction ($P < 0.001$) for the effect of i.c.v. kisspeptin on FSH secretion (Fig. 1). Mean concentration, peak concentration, and AUC of serum FSH were increased ($P < 0.001$) in period 2 for kisspeptin-treated animals in a dose-dependent manner when compared with control animals (Fig. 3). Serum FSH concentrations were increased ($P < 0.05$) in control animals following GnRH treatment in period 3 (Fig. 1). Serum concentrations of FSH had not returned to baseline levels in the kisspeptin-treated animals at the beginning of period 3 (Fig. 1), thus there was no difference in the parameters of FSH secretion between control and kisspeptin-treated animals following GnRH infusion (Fig. 3).

An expected variation in secretory patterns of GH between animals was observed; however, serum

Figure 3 Mean FSH, peak FSH, and area under the curve (AUC) of serum FSH for prepubertal gilts receiving i.c.v. infusion of saline or either 10 or 100 µg kisspeptin (Kiss). Data in period 1 are from the 3 h prior to i.c.v. treatment; data in period 2 are from the 3 h after i.c.v. treatment; and data in period 3 are from the 90 min following an i.v. infusion of 100 µg GnRH. Data are presented as mean $\pm$ S.E.M. ^{a,b}Means within a period differ between treatments ($P < 0.05$).

concentrations of GH were not affected by i.c.v. treatment (Fig. 4). Mean concentrations of GH in serum following i.c.v. infusion were 4.15 ± 1.9 , 2.33 ± 0.5 , and 2.96 ± 1.1 ng/ml for controls, Kiss 10 µg, and Kiss 100 µg treated animals respectively.

Experiment 2

There was a treatment–time interaction ($P < 0.001$) for serum concentrations of LH. Serum LH concentrations increased following i.v. infusion in all kisspeptin-treated animals (Fig. 5). Compared with period 1, mean concentration, peak concentration, and AUC of serum LH of control animals were unchanged in period 2 but were greater ($P < 0.05$) for all kisspeptin-treated animals (Fig. 7). Mean LH for animals treated with 5 mg kisspeptin tended ($P < 0.1$) to be greater than for animals treated with 1 mg kisspeptin (Fig. 7). Area under the LH curve increased ($P < 0.05$) in a linear fashion with increasing dose of kisspeptin (Fig. 7), which resulted from more sustained release of LH following the higher doses (Fig. 5).

Figure 4 Serum GH concentrations for prepubertal gilts receiving i.c.v. infusion of saline or either 10 or 100 µg kisspeptin (Kiss). Data of three representative animals from each treatment are shown. The dashed vertical line indicates the time at which i.c.v. treatments were administered. There was no treatment–time interaction detected.

Intravenous infusion of kisspeptin at the highest dose tended ($P=0.08$) to increase serum concentrations of FSH at 45–90 min after treatment (Fig. 6). There was neither a treatment nor treatment–period interaction on parameters of FSH secretion (Fig. 7). However, the overall mean in period 2 was greater ($P<0.05$) for peak FSH (2.29 ± 0.18 vs 2.56 ± 0.18 ng/ml for periods 1 and 2 respectively) and AUC of FSH (343 ± 33 vs 395 ± 33 ng/ml for periods 1 and 2 respectively) than in period 1. Concentrations of GH in serum were similar for all animals and were unchanged by i.v. infusion of 1, 2.5, or 5 mg kisspeptin (Table 1).

Discussion

We report here for the first time that central infusion of kisspeptin to prepubertal gilts rapidly induces LH secretion. This strongly supports the concept that the role kisspeptin has in regulating the onset of puberty in the rodent (Navarro *et al.* 2004a, 2004b, Castellano *et al.* 2006) and primate (Plant *et al.* 2006) may be conserved in the domestic pig, which is an important biomedical model (Tumbleson 1986, Lunney 2007). The AUC, which reflects the integrated pattern of LH secretion, was increased by kisspeptin in a dose-dependent manner. The 10 µg dose (7.5 nmol) of kisspeptin resulted

Figure 5 Serum LH concentrations (mean $\pm$ S.E.M.) for prepubertal gilts receiving i.v. infusions of saline or either 1, 2.5, or 5 mg kisspeptin (Kiss). A treatment–time interaction was detected ($P<0.01$). *Times at which effects of treatment were different from saline-treated control animals ($P<0.05$).

Figure 6 Serum FSH concentrations (mean $\pm$ S.E.M.) for prepubertal gilts receiving i.v. infusions of saline or either 1, 2.5, or 5 mg kisspeptin (Kiss). A tendency for a treatment-time interaction was detected ($P=0.08$). #Times at which effects of treatment tended to be different from saline-treated control animals ($P<0.10$).

in a rapid pulse of LH that gradually declined over the next 3 h, whereas the pulse of LH induced by 100 μ g (75 nmol) kisspeptin persisted throughout the sampling period. Presumably, this reflects the kisspeptin-induced pattern of GnRH secretion. Kisspeptin has been shown to

stimulate the *in vitro* release of GnRH from hypothalamic explants of rats (Thompson *et al.* 2004), and central infusion of ovariectomized ewes with 50 nmol kisspeptin over a 4-h period caused the sustained secretion of GnRH into the cerebrospinal fluid

Figure 7 Mean concentration, peak concentration, and area under the curve (AUC) of serum LH and FSH for prepubertal gilts receiving i.v. infusion of saline or either 1, 2.5, or 5 mg kisspeptin (Kiss). Data in period 1 are from of the 3 h prior to treatment and data in period 2 are from the 3 h after treatment. Data are presented as mean $\pm$ S.E.M. a,b,c Means within a period differ between treatments ($P<0.05$).

Table 1 Effect of i.v. administration of kisspeptin (Kiss) on serum concentrations of growth hormone (GH; ng/ml) in prepubertal gilts.

Period ^a	Treatment				S.E.M. ^b	P value		
	Control	Kiss 1 mg	Kiss 2.5 mg	Kiss 5 mg		Treatment	Period	Treatment – period
1	3.11	3.17	2.92	3.90	0.39	0.16	0.56	0.70
2	2.56	3.23	2.74	4.03	0.47			

^aPeriod 1, before treatment; period 2, after treatment. ^bS.E.M., overall, S.E.M. within treatment.

concomitantly with elevated serum concentrations of LH (Messenger *et al.* 2005). We cannot preclude, however, that the pattern of LH secretion observed here might be caused by alterations in GnRH pulse frequency or amplitude. Whether or not kisspeptin regulates the GnRH pulse generator is not presently known. Regardless, pituitary function was intact in these animals as indicated by the increased LH secretion in control pigs following an i.v. infusion of GnRH in period 3. The GnRH-induced release of LH in animals that had received i.c.v. infusions of kisspeptin was reduced when compared with that of the control animals that had received i.c.v. infusion of saline. In view of the prolonged stimulation of LH release following kisspeptin treatment, this reduced response to GnRH likely reflects a reduction in the releasable pool of LH.

In order to determine whether peripheral administration of kisspeptin could induce LH secretion in prepubertal pigs, we administered an i.v. bolus of kisspeptin at three different doses. All three doses tested were able to elevate secretion of LH in these animals. This is substantiated by reports in rodents and monkeys which demonstrate kisspeptin stimulates LH secretion when given either centrally or peripherally (Matsui *et al.* 2004, Navarro *et al.* 2005b, Shahab *et al.* 2005). At the time our study was conducted, it was reported that i.v. administration of <3 mg kisspeptin to ovariectomized ewes resulted in somewhat variable LH release (Arreguin-Arevalo *et al.* 2007). Since the completion of our study, it has now been shown that i.v. administration of kisspeptin at doses of <1 mg can reliably elicit LH secretion, at least in the ovariectomized estradiol-treated ewe (Caraty *et al.* 2007). In the present study, the magnitude of LH release was similar for all doses of kisspeptin but the duration was longer with increasing dose. Whether or not i.v. infusion of kisspeptin at <1 mg can reliably induce LH secretion in the pig warrants further investigation.

Information regarding the effects of kisspeptin on FSH secretion is limited. We demonstrate that the central administration of kisspeptin to prepubertal gilts increased serum concentrations of FSH. This effect of kisspeptin on FSH secretion in the pig is in agreement with reports in rodents (Matsui *et al.* 2004, Thompson *et al.* 2004, Navarro *et al.* 2005a) and primates (Plant *et al.* 2006). The increase in FSH induced by kisspeptin showed more gradual onset and the magnitude was less than that of LH. The ED₅₀ of

kisspeptin to release FSH has been estimated to be ~100 times higher than that for LH in rats (Navarro *et al.* 2005a). This may reflect the fact that FSH is not wholly under the control of GnRH (Kile & Nett 1994, Phillips 2005). Despite a clear effect of i.c.v.-infused kisspeptin on FSH secretion, i.v. infusion only tended to alter FSH secretory patterns in prepubertal gilts infused with the highest dose of kisspeptin. This is similar to observations in the ovariectomized ewe (Arreguin-Arevalo *et al.* 2007). However, it was recently reported that doses of kisspeptin up to tenfold less than used in our experiment were able to stimulate FSH secretion in ovariectomized estradiol-treated ewes (Caraty *et al.* 2007). Differences in species and physiological status of the animals may account for differing results when kisspeptin is infused intravenously.

Concentrations of FSH in serum remained elevated at the end of the 3 h sampling period in the animals receiving i.c.v. infusion of 100 µg kisspeptin, despite the fact that LH in those animals was declining. Interestingly, a similar observation has recently been reported in ovariectomized estradiol-treated ewes (Caraty *et al.* 2007). In the present experiment, we were unable to determine whether this is due to a true change in secretion, or to the longer half-life of FSH (Macdonald *et al.* 2007). In the pig, circulating concentrations of LH are reduced more rapidly than those of FSH after a bolus GnRH infusion (Wise *et al.* 1996). Nonetheless, the initial pattern of FSH secretion induced by i.c.v. infusion of kisspeptin is similar to that caused by i.v. infusion of GnRH in the control animals. This supports the concept that the primary mechanism of kisspeptin-induced FSH secretion is mediated through the GnRH neuronal network (Thompson *et al.* 2004, Irwig *et al.* 2005, Messenger *et al.* 2005).

During preparation of this manuscript, it was reported that i.v. administration of kisspeptin stimulated GH secretion in prepubertal heifer calves (Kadokawa *et al.* 2008). Indeed, age-related changes in serum concentrations of GH occur in the pig but typically decline as the animal is approaching puberty (Machlin *et al.* 1968, Klindt & Stone 1984, Dubreuil *et al.* 1987). We found no effect of either central or peripheral administration of kisspeptin on secretion of GH in the present study. Divergence with regard to kisspeptin's actions on GH secretion may be related to species differences. However, our experiment included a true control group, which may have allowed us to more

accurately delineate the effect of kisspeptin on GH secretion in the pig. The inclusion of saline-treated controls clearly illustrate that, in our hands, kisspeptin does not modulate the secretion of GH in the prepubertal pig, and agrees with observations in monkeys (Plant personal communication).

In conclusion, here we report that kisspeptin can activate the gonadotropic axis in the prepubertal pig. The ability of kisspeptin to stimulate hormone secretion from the anterior pituitary gland of the pig seems to be specific for LH and FSH and did extend to GH in this study. We presently do not know whether other anterior pituitary hormones in the pig are affected by kisspeptin. Our data are consistent with the idea that kisspeptin's actions on gonadotropin secretion are mediated primarily through modulating secretion of GnRH and support the concept that kisspeptin plays an important role in regulating puberty.

Materials and Methods

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of The University of Georgia. Prepubertal gilts (sow line C42, boar line 280; PIC, Franklin, KY, USA) weighing 67.6 ± 7.2 kg (mean $\pm$ s.d.) and 130-day age were used. Animals were born and reared at the Swine Research Center at The University of Georgia. During experiments, animals were moved to a Large Animal Research Unit, where they were kept in individual pens under controlled temperature (22 °C) and had access to feed and water *ad libitum* unless otherwise noted. The diet was formulated to meet National Research Council guidelines (NRC 1988) for growing swine. Kisspeptin consisted of the ten C-terminal amino acids of the murine peptide and was obtained from GenScript Corp. (Scotch Plains, NJ, USA). GnRH was obtained from Sigma or Merial Limited (Cystorelin; Iselin, NJ, USA).

Experiment 1

Animals ($n=14$) were surgically fitted with a lateral i.c.v. cannula using a stereotaxic procedure described previously (Estienne *et al.* 1990, Barb *et al.* 1993). Placement of the i.c.v. cannula of each animal was verified by X-ray. At least 1 week after the placement of i.c.v. cannula, and 24 h prior to treatment, all animals were fitted with indwelling jugular catheters (Barb *et al.* 1982). Animals were randomly assigned to one of three groups. Control animals received 150 μ l PBS. The other groups received either 10 or 100 μ g kisspeptin in 150 μ l PBS. Doses of kisspeptin were based on our previous experience with i.c.v. administration of hormones to the pigs (Barb *et al.* 2004, Barb & Barrett 2005). Serial blood samples were drawn every 15 min for 3 h before (period 1) and 3 h after (period 2) i.c.v. treatment, and for 90 min (period 3) following an i.v. bolus infusion of 100 μ g GnRH. The time from the end of period 2 to the beginning of period 3 was 15–45 min. The inclusion of the GnRH treatment was to verify that pituitary function was intact and that LH secretion could in fact be

induced in these prepubertal gilts. One week later, the experiment was replicated with animals reassigned to treatment so that no animal received the same treatment a second time, resulting in eight pigs for the control treatment and ten pigs for each dose of kisspeptin. Gilts were killed and the ovaries were subjected to gross inspection to confirm the absence of luteal structures.

Experiment 2

Experiment 2 was conducted with six prepubertal gilts in each treatment group to determine the response to increasing doses of kisspeptin administered as a single bolus i.v. infusion. Animals were fitted with indwelling jugular catheters the day prior to the experiment. Animals received 1, 2.5, or 5 mg kisspeptin in 3 ml PBS or 3 ml PBS alone for the control animals. Doses were chosen in part based on the reported effective dose in sheep (Arreguin-Arevalo *et al.* 2007). At 0730 h, feeders were removed from pens and blood sampling started at 0800 h. Serial blood samples were drawn every 15 min for 3 h before (period 1) and 5 h after (period 2) treatment. Feeders were returned to all pens after the last blood sample was drawn.

Hormone analysis

Blood was allowed to clot for 1 h at room temperature and then 4 °C overnight. Serum was separated by centrifugation and stored at -20 °C for subsequent analysis of LH (Kesner *et al.* 1987) and FSH (Trout *et al.* 1992) by RIA. The reference standard for LH (AFP-10506A) and FSH (AFP-10640B) was provided by Dr A F Parlow, Scientific Director of the NIH, NIDDK, National Hormone and Peptide Program. Sensitivity of the assays was 0.15 and 0.2 ng/ml for LH and FSH respectively. Intra- and inter-assay coefficient of variation (CV) of LH assays were 7.9 and 9.8% respectively. Four pools of porcine serum with FSH concentrations that ranged from 1 to 3 ng/ml were included in each assay; these had intra-assay CV that ranged from 4 to 16%. Serum concentrations of GH were determined using a porcine RIA kit (Millipore Corp., Billerica, MA, USA). Sensitivity of the assay was 1 ng/ml and intra- and inter-assay CV were 8.6 and 13.4% respectively.

Statistical analysis

To determine the effect of kisspeptin on serum concentrations of LH, FSH, and GH, data were subjected to generalized least squares ANOVA with repeated measures using the MIXED procedure of SAS (1999). The model included replicate, treatment, time, and all first- and second-order interactions, with a compound symmetric function used to model the covariance structure for the repeated measures. If a significant ($P<0.05$) treatment–time interaction was detected, the simple effects of treatment within a time were compared using the SLICE option of the LSMEANS statement of SAS. Mean concentration, peak concentration, and AUC of serum LH and FSH at fixed periods were subjected to generalized least squares ANOVA with repeated measures. In experiment 1,

periods 1 and 2 were defined as the 3 h before and 3 h after i.c.v. treatment respectively. Period 3 was defined as the 90 min following i.v. infusion of GnRH. In experiment 2, periods 1 and 2 were defined the same as in experiment 1. The model included replicate, treatment, period, and all first- and second-order interactions, with a compound symmetric function used to model the covariance structure for the repeated measures.

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