



OPEN Intertwining of the IGF system and animal welfare

Anne-Marie Galow¹✉, Daniela Ohde¹, Anja Eggert¹, Christina Walz¹, Marianne Zenk¹, Chaithra Umesh², Saptarshi Bej³, Olaf Wolkenhauer² & Andreas Hoeflich¹✉

To date, husbandry categories are defined based on the human concept of animal needs, however, to objectively assess animal welfare in defined husbandry systems, animal-related indicators must be evaluated. Here, we studied the impact of differential housing conditions on factors of the insulin-like growth factor system (IGF-system) in mother sows either housed in a conventional husbandry system corresponding to the regulations of the German husbandry form 1, or in the associated ecological facility, corresponding to the German husbandry form 4/5. Utilizing a proprietary bioassay that determines the activity of the IGF system at the level of intracellular signal transduction, we revealed altered serum IGF bioactivities in differentially housed sows. Moreover, we present a comprehensive analysis of the individual factors of the IGF system in serum of pregnant and lactating sows to elucidate which of these are modified depending on the housing conditions. An unsupervised principal component analysis suggests that IGF system factors might distinguish sows based on their housing environment. These results imply that the IGF system provides a solid basis for the documentation and objective assessment of animal health and welfare. However, a robust biomarker system for animal welfare assessment will likely require a multifactorial and integrative approach.

Keywords Pig farming, Housing system, Stress, Cortisol, Biomarker, IGF1

Abbreviations

BCA	Bicinchoninic acid
BIRA	IGFBP2-enhanced IGF-related AKT activation
Dpc	Days post coitum
Dpp	Days post partum
EMEM	Eagle's minimal essential medium
EMM	Estimated marginal means
ERK	Extracellular-signal regulated kinase
FBS	Fetal bovine serum
GABA	Gamma-aminobutyric acid
GH	Growth hormone
IGF	Insulin-like growth factor
IGFBP	IGF binding protein
IGF1R	IGF1 receptor
IQR	Interquartile range
KNN	K-nearest neighbors
LMM	Linear mixed-effects model
MAPK	Mitogen-activated protein kinase
PAGE	(SDS)-polyacrylamide gel electrophoresis
PAPP-A	Pregnancy-associated plasma protein A
PBS	Phosphate-buffered saline
PCA	Principal component analysis
REML	Restricted maximum likelihood
SDS	Sodium dodecylsulfate
STC	Stanniocalcin
TBST	Tris-buffered saline with Tween20

¹Research Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, Dummerstorf 18196, Germany.

²Department of Systems Biology and Bioinformatics, University of Rostock, Rostock 18051, Germany. ³School of Data Science, IISER Thiruvananthapuram, Vithura 695551, Kerala, India. ✉email: galow@fbn-dummerstorf.de; hoeflich@fbn-dummerstorf.de

The increasing interest of consumers in the farming conditions of animals, motivates considerations about animal welfare labeling as discussed in the framework of the EU Farm to Fork Strategy. In Germany, voluntary labeling, based on different husbandry forms concerning the type of shelter, available space, and opportunities to express natural behaviors, has already been introduced (husbandry forms 1–5)¹. However, these categories are defined based on the human concept of animal needs; to assess whether animals actually benefit from the superior husbandry forms, animal-related indicators must be recorded and evaluated. The Welfare Quality⁷ Assessment Protocol² is one attempt to assess animal welfare but it only records externally recognizable characteristics such as behavioral disorders, diseases or injuries and does not include any laboratory tests in stages before adverse manifestations. However, in order to be able to take timely action, it must be achieved to detect discomfort and stress before the onset of mental and physical damage.

To date, hormones of the pituitary-adrenal axis, such as cortisol, are commonly used as biomarkers, particularly for acute stress. However, these hormones are less suitable as indicators of long-term stress and well-being since they are characterized by relatively short half-lives and a pulsatile secretion usually following circadian and seasonal rhythms, which generally necessitates repeated measures³. More importantly, several lines of evidence suggest that chronic stress in pigs might not even be effective in deflecting basal cortisol levels⁴. Even if the triggering stimulus is maintained, plasma cortisol levels usually decline after the acute response. For instance, pigs receiving unpredictable and inescapable electric shocks for a month have plasma cortisol levels similar to those of control animals not receiving any shock. Even though, they still react behaviorally to the situation (agitation followed by inhibition), suggesting that they are continuously stressed⁵. While hair cortisol is suggested to better represent chronic stress, there are still several practical limitations such as local and external sources of cortisol “contaminating” the hair and differences in hair growth rate within the same body region that could affect hair cortisol concentrations⁶. Taken together, hormones of the pituitary-adrenal axis, such as cortisol, are not well suited to assess animal welfare in dependence on the husbandry conditions, particularly in pigs.

The pituitary-liver axis (somatotrophic axis), which initially induces growth hormone (GH) and subsequently controls the insulin-like growth factor (IGF) system, is also regulated by stress⁷. Here, we propose that the activity of this system is informative not only for short- and medium-term stress, as we have already shown^{8,9}, but also for longer-term stress and well-being on the other hand.

The IGF system includes two forms of insulin-like growth factors (IGF1 and IGF2), their receptors, and IGF-binding proteins (IGFBPs) that regulate the bioavailability of IGFs¹⁰. Additionally, its activity is modulated by several other factors. For example, the binding of IGFBPs is regulated by proteases such as PAPP-A and PAPP-A2, which are in turn modulated by inhibitors such as stanniocalcin 1 and 2 (STC1 and STC2)^{11,12}. Recently, it was shown that also calcium affects IGF signaling¹³. Figure 1 provides an illustration of these several layers of IGF bioactivity modulation. The IGF1 receptor (IGF1R) belongs to the receptor tyrosine kinase family and induces pathways such as PI3K/AKT/mTOR and MAPK/ERK. Our proprietary BIRA assay is based on the detection of AKT phosphorylation to assess IGF-related bioactivity downstream of the IGF1R, thereby accounting for the integration of multiple signals exerted by free IGFs, IGFBPs, IGFBP-proteases, and protease inhibitors¹⁴.

Here, we consider both, the overall IGF-related bioactivity as well as quantities of individual factors to investigate the impact of the environment, and welfare aspects in particular, on the IGF system. More generally, we aim to clarify if the husbandry categories for pigs, defined by humans, represent distinct levels of welfare that can be measured objectively. To approach this question, we tested the feasibility of discriminating between husbandry systems based on selected candidate parameters utilizing unsupervised principal component analysis (PCA).

Methods

Housing conditions in conventional and ecological facility

For the study, German landrace sows were housed in the experimental animal facility at FBN Dummerstorf in compliance with European animal welfare regulations for appropriate husbandry and experimental conditions as well as the national Animal Welfare Act and Animal Protection Farm-Animal-Husbandry-Ordinance (German TierSchNutzV). The animals were divided in two experimental groups, one being housed in the conventional husbandry system corresponding to the definition of the German husbandry form 1, and the other being housed in the associated ecological (eco) facility, corresponding to the definition of the German husbandry form 4/5 (Fig. 2). The housing experiment involved the impregnation of the sows and consecutive rearing of up to three litters. For each parity, the sows age at insemination is given in Supplementary Table S1 and S3. The sows were mated with 10 different boars, covering individuals from both husbandry systems. Mating assignments were determined by the institute's pig herd manager, without external influence. On the 105th day of gestation, the sows were moved from group pens into the farrowing pens.

For the conventional husbandry, pregnant sows were housed in three-area pens (walking area - concrete slatted floor, lying area - flat paved with litter, feeding stalls - self-catching stalls with partially slatted floor) for 4 sows each with an area of 22 m² for old sows and 15.9 m² for gilts (corresponds to a pen area of 5.5 m² per old sow and 4.0 m² per gilts). Animals were fed twice a day with a complete feed for pregnant sows (Sauen Struktur tragend, Trede and von Pein, Itzehoe, Germany), according to the recommendations of the Society for Nutritional Physiology for the energy and nutrient supply of pigs¹⁵. The animals were provided with enrichment material in the form of long straw in racks, shavings and spelt litter as well as chains with wood. Water was accessible at all times via a drinking trough. The farrowing pen offered the sow and suckling piglets an area of 6.5 m², while allowing for the restraint of the sows from the 114th day of pregnancy until the piglets were four days old. The pens are equipped with plastic slatted floors, cast-iron elements in the sow's lying area and a piglet nest (0.75 m²) with a climate cover and a heat lamp. The sows were fed twice daily as required (gradually increasing the daily feed quantity from 1 kg after birth to 7–8 kg 14 days post partum) with a complete feed for

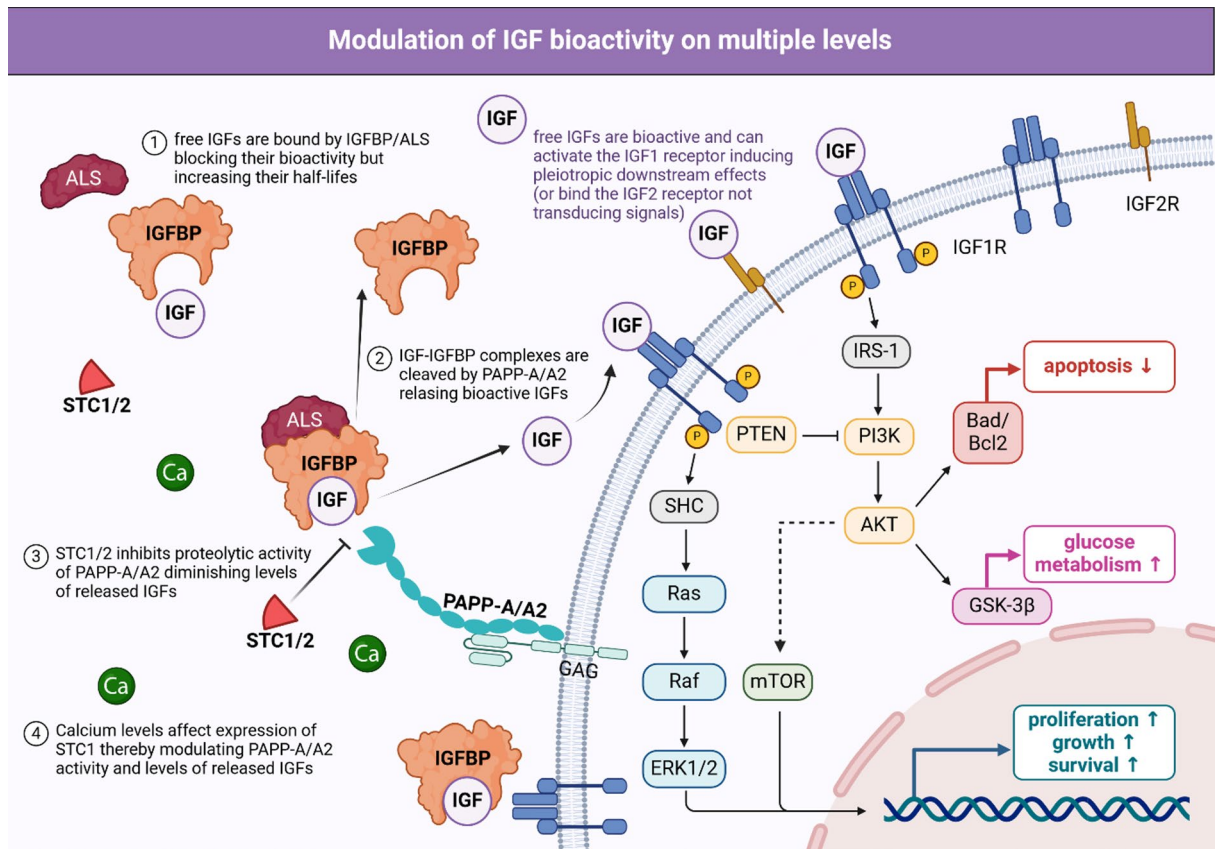


Fig. 1. Graphical summary of factors affecting IGF bioactivity.

lactating sows (Sauen Struktur säugend, Trede and von Pein, Itzehoe, Germany). In addition to the bedding mix, wood on chains was provided as enrichment material. The birth was induced by administering 2 ml PGF Veyx (Cloprostenol, Prostaglandin F_{2α}) one day before the calculated day of birth and 1 ml Longacton (Carbetocin, Oxytocin) on the day of birth.

In the eco facility, the group pen offers a stall area of 66 m² and an exercise area of 45 m² housing 8–16 sows during the experiment. This corresponds to a space of minimally 4.1 m² stall area plus 2.8 m² outside exercise area per sow. The animals are fed a high-fiber, GMO-free, organic feed (B Sauenfutter NT Pell., Cera-Green) via a call-off station. Fixed amounts of feed (depending on their condition and stage of pregnancy) can be called off throughout the day and water is available ad libitum via two trough drinkers. The pens are littered with straw in all areas and an automatic cattle brush is provided as an additional activity material. The farrowing pens offer approx. 8.4 m² of littered inside pen space and 5 m² of outside area. A piglet nest (0.75 m²) with underfloor heating and a heat lamp is integrated into the pen. The sows and piglets can move freely in the pen, but the sows can be restrained for short periods. The sows are fed a high-fiber, nutritionally balanced organic lactation feed (B Sauenfutter LAC Pell., CeraGreen) several times a day following the recommendations of the Society for Nutritional Physiology for the energy and nutrient supply of pigs¹⁵. Composition and nutritional contents of all animal feed is declared in Supplemental Table S2. Water is available to the animals at all times via a drinking trough and a mother-child drinking trough ad libitum. There is no routine induction of labor with prostaglandin and/or oxytocin.

Monitoring of general health parameters of mother sows and offspring

Generally, all animals are cared for and looked after by experienced staff of the experimental animal facility at FBN recording any abnormalities that indicate illness. For the litters in particular, a number of parameters are routinely recorded, such as the total number and gender of the piglets and their birth weight. Moreover, any abnormalities such as the occurrence of still born piglets, piglets with defective leg muscles (splay leg cases), and low weight and feeble animals are documented (see Supplementary Table S1). Of note, while all piglets per litter were considered for the ratio of males and the ratio of still born piglets and splay leg cases, only piglets born alive were considered for the calculation of the mean birth weight and ratio of low-birth-weight piglets in this study.

Sampling of blood and saliva

At day 30 and 105 of gestation as well as day 8 after farrowing, approximately 10 ml of blood were collected from each sow in 50 ml tubes by puncture of the cranial vena cava under fixation. For serum preparation, whole blood was centrifuged at 1.500 g for 10 min. To improve the purity of the samples, the supernatant was transferred to

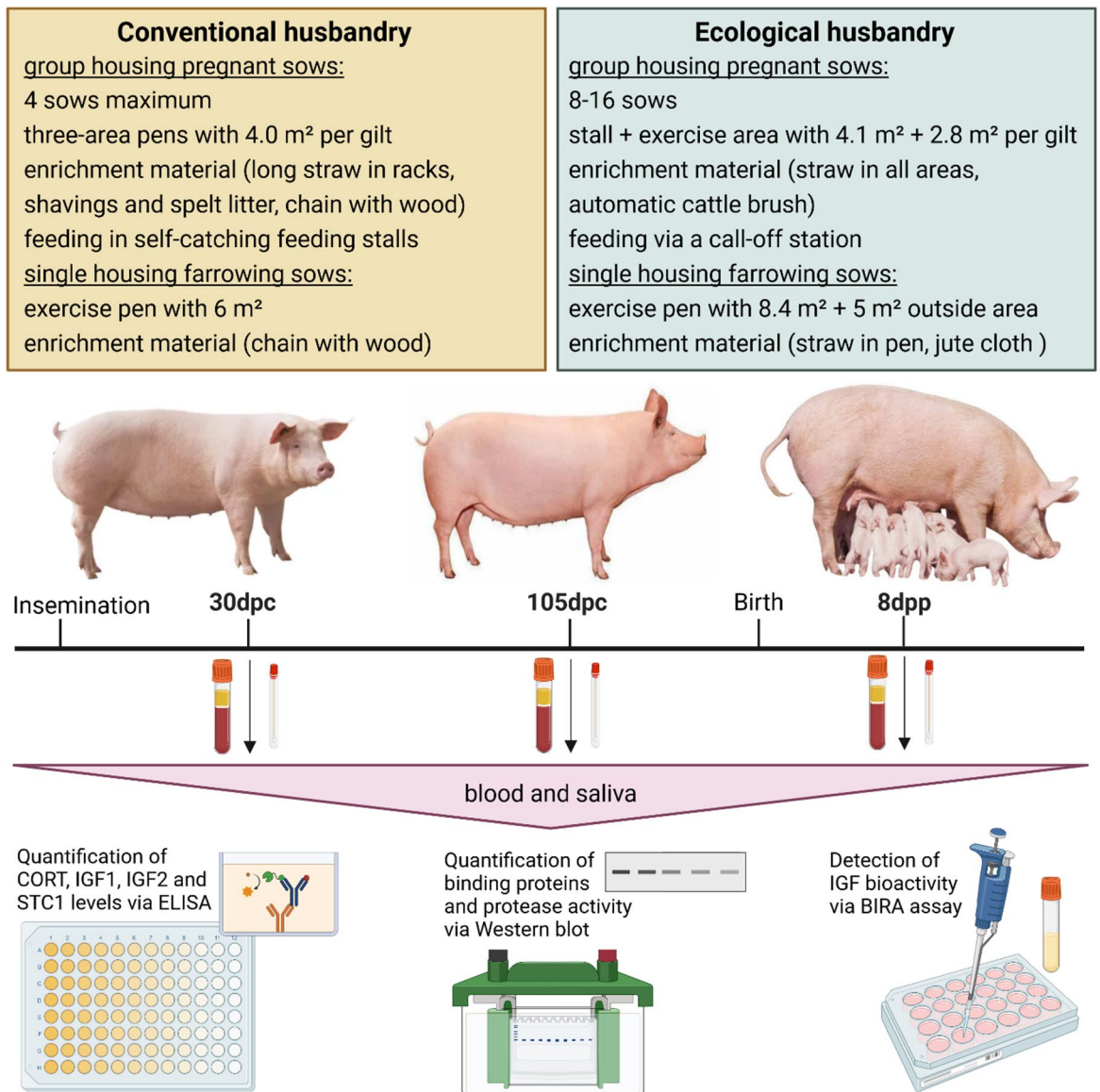


Fig. 2. Experimental design to study effects of the housing environment on factors of the somatotrophic axis. Blood and saliva samples were obtained from differentially housed sows 30 and 105 days post coitum (dpc) as well as 8 days post partum (dpp) to analyze abundance and biological activity of candidate factors (CORT: cortisol).

new tubes and centrifuged again at 1.500 g for 5 min before the serum was transferred to cryo-tubes and stored at -80°C until usage.

Additionally, non-invasive sampling of saliva using swabs (5001.06, Salimetrics, Carlsbad, USA) was conducted before blood sampling. For this, the swabs were kept in the sows' mouths for at least one minute until satisfactory moistening was achieved. To collect the saliva, the swabs were placed in syringe without cannula and put in 15 ml tubes for a centrifugation at 2.000 g for 10 min. Saliva was then transferred in cryo-tubes and stored at -80°C until usage. Samplings were generally conducted in the morning (7:00–7:30). Serum and saliva samples were used for a number of assays as outlined in the following. An overview of the experimental design is illustrated in Fig. 2.

Quantification of cortisol concentrations

For the determination of cortisol concentrations in serum and saliva, respectively, commercial ELISA Kits (DEH3388 and DES6611, demeditec, Kiel, Germany) were used according to the instructions of the manufacturer. Briefly, serum samples were homogenized by vortexing, while saliva samples were diluted 1:2 in sample buffer and centrifuged at 2.000 g for 3 min to pellet any contaminants. 10 μl (for serum) and 50 μl (for saliva) of each sample, control and calibrator were dispensed in duplicates in the anti-cortisol antibody coated 96-well plate. 200 μl and 50 μl enzyme conjugate, respectively, were added to the wells and incubated for 1 h at room temperature

under gentle shaking followed by several washing steps. Subsequently, 200 μl of substrate buffer was pipetted and incubated for 30 min at room temperature in the dark for both analyzed matrices. Finally, 50 μl of stop solution were added to each well and absorbance was measured at 450 nm. Cortisol concentrations were calculated based on a four-parameter logistics curve fit of the calibrator values. For saliva samples, protein concentrations were determined using BCA (Bicinchoninic acid) tests to normalize the obtained results regarding potential dilution effects due to variations in salivation and drinking behavior of the sows.

Determination of IGF-related bioactivity - BIRA assay

For the BIRA (IGFBP2-enhanced IGF-related AKT activation) assay we used a transfected human embryonic kidney fibroblast cell line (HEK293), obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). HEK293 cells were transfected with 8 μg pCMV-int-mIGFBP2 (XhoI linearized) and 0.8 μg EcoRI linearized neomycin resistance plasmid pSV2neo (Clontech, Heidelberg, Germany)¹⁶, which renders the cells more sensitive to bioactive IGF¹⁴. HEK293 clones were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS; 10500-064; Gibco, Carlsbad, CA, USA) and 1% 100 \times penicillin/streptomycin/amphotericin B mixture (882087; Lonza, Gampel, Switzerland) at 37 °C and 5% CO₂.

To prepare the bioassay, cells were detached using 0.25% trypsin in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and seeded in 24-well-plates with 1–2 \times 10⁵ cells per well in 500 μL cell culture medium. After reaching 90% confluence, medium was exchanged to EMEM with 0.5% FBS for 16–20 h to reduce triggering stimuli originating from the serum. After the cells were washed with PBS, 240 μl of serum samples (diluted 1:10 in PBS) and serial dilutions from 6.25 ng/ml to 100 ng/ml of human recombinant IGF1 (Cat. #100–11, PeproTech, Inc., Rocky Hill, NJ, USA), were added for 20 min at 37 °C. Subsequently, the test media were discarded and cells were washed twice with PBS to eliminate any protein residues, before the cells were lysed in 100 μL lysis buffer (150 mM NaCl, 50 mM Tris/Cl, 1% Tween 20, Complete Mini protease inhibitor cocktail dissolved in VE water).

For sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), 100 μl 2 \times Laemmli (125 mM Tris(hydroxymethyl)-aminomethane, 4% SDS, 20% glycerine) containing 0.01% bromophenol blue and 4% beta-mercaptoethanol were added before lysates were homogenized for 30 min at 45 °C in an ultrasonic bath. Samples were denatured at 95 °C for 5 min prior to PAGE utilizing the Bio-Rad TGX Stain-Free FastCast Acrylamide-Kit (Bio-Rad Laboratories GmbH, Munich, Germany). All gels were blotted onto polyvinylidene fluoride membranes (PVDF, pore size 0.45 μm , Carl Roth GmbH + Co. KG, Karlsruhe, Germany), blocked for 1 h with 3% milk powder in Tris-buffered saline with Tween20 (TBST) and incubated overnight with a primary antibody for AKT phosphorylated at serine 473 (CST #4058, dilution 1:1000, Cell Signaling Technologies). After washing, the membranes were incubated for 2 h with a secondary antibody (antirabbit IgG HRP, CST #7074, dilution 1:2000, Cell Signaling Technologies). Bands were visualized using Lumigen ECL Ultra (Lumigen Inc., Southfield, MI, USA) in a Bio-Rad Chemi-Doc MP system (Bio-Rad Laboratories GmbH). Images were analyzed using Image Lab Software Ver. 6.0.1 (Bio-Rad Laboratories GmbH, Hercules, CA, USA) and signal intensities normalized to total protein concentration per lane. Finally, values of the IGF1 standards were used to determine IGF1 equivalents in ng/ml for all samples. However, since for complex matrices like serum, phosphorylation of AKT cannot be translated into IGF1 concentrations in ng/ml directly, we used the mean values for standard housing at 30 dpc as reference value to calculate a relative IGF-dependent bioactivity for the investigated samples from differentially housed pregnant (105 dpc) and lactating (8 dpp) sows.

Quantification of IGF1 and IGF2 concentrations

For the determination of total IGF1 and IGF2 concentrations in serum commercial ELISA Kits (E20 and E30, Mediagnost, Reutlingen, Germany) were used according to the manufacturers protocols. In short, serum samples were briefly vortexed for sample homogenization and diluted in sample buffer (1:21 for IGF1, 1:404 for IGF2). Two serum samples with known IGF concentrations each were included for assay quality control and prepared similarly. The microtiter plate was prepared with 80 μl /50 μl antibody conjugate before 20 μl /50 μl of samples, controls, blanks and calibrators were added to each well and incubated for 1 h/2 h at room temperature under gentle shaking. After multiple washing steps, 100 μl of enzyme conjugate was added for 30 min at room temperature under gentle shaking. Following further washing steps, 100 μl of substrate buffer was pipetted and incubated for 15 min at room temperature in the dark, before 100 μl of stop solution was added to each well. Absorbance was measured at 450 nm and IGF concentrations were calculated based on a third-degree polynomial curve fit of the calibrator values.

Determination of IGFBP concentrations

Serum samples and serial dilutions of recombinant human IGFBP standards (R&D Systems, Wiesbaden, Germany) were diluted 1:20 and boiled in sample buffer [312.5 mM Tris (pH 6.8), 50% (w/v) glycerol, 5 mM EDTA, 1% (w/v) SDS, and 0.02% bromophenol blue] for 5 min. Proteins were separated by 12% SDS-PAGE using TGX Stain-Free™ FastCast™ acrylamide kit (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) and transferred to a PVDF membrane (Millipore, Bedford, USA). The blots were blocked with 1% fish gelatine in TBST and then incubated with biotin labeled human IGF2 (1:500; BioIGF2-10; ibt-systems, Binzwangen, Germany), followed by streptavidin conjugate incubation and a detection step with Lumigen ECL Ultra (TMA-6) (Lumigen, Southfield, USA). Bands were visualized on ChemiDoc XRS + gel imaging system (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) and quantified using Image Lab software Ver. 6.0.1 (Bio-Rad Laboratories GmbH, Hercules, CA, USA). Signal intensities were normalized using stain-free gel images and IGFBP concentrations were quantified using the standard serial dilutions. For each sample two technical replicates were generated on independent gels. If the results of technical replicates differed considerably, two additional technical replicates were generated and average values were used for downstream analyses.

Detection of protease activity

To test for proteolytic cleavage of IGFBBPs in serum, samples were diluted 1:5 in PBS and incubated in a thermal shaker at 39 °C and 40 rpm for up to 44 h. At different time points (4, 20, 28 & 44 h), samples were mixed and centrifuged, an aliquot was taken and thoroughly mixed with an equal amount of 2x sample buffer (312.5 mM Tris (pH 6.8), 50% (w/v) glycerol, 5mM EDTA, 1% (w/v) SDS, and 0.02% bromophenol blue). Samples without incubation served as positive controls. Detection of intact IGFBBPs followed the protocol for SDS-PAGE and Western ligand Blotting as described above for the “Determination of IGFBP concentrations”.

Quantification of STC1 concentrations

For the quantitative detection of STC1 levels in serum a commercial ELISA kit (EK1404, Boster Biological Technology, Pleasanton, USA) was used according to the manufacturer’s protocol with some adjustments accounting for the highly variable STC1 concentrations recognized in a pilot experiment. For a wide concentration range of standards, a 10ng/ml stock solution was diluted to 5000, 2000, 1000, 500, 250, 125, 62.5 and 0 pg/ml. 100 µl of standards and serum samples were pipetted in duplicates in a 96-well plate and incubated for 1.5 h at 37 °C. After discarding the fluids, 100 µl of a biotinylated polyclonal STC1 antibody was added per well and incubated for 1 h at 37 °C. After washing, 100 µl per well of an avidin-biotin-peroxidase complex were pipetted and incubated for 30 min at 37 °C. Following further thorough washing steps, 90 µl color developing reagent per well was pipetted and kept for a reduced incubation time of 20 min at room temperature in the dark, before 100 µl stop solution were added. Absorbance was measured at 450 nm and STC1 concentrations were calculated based on a four-parameter logistics curve fit of the standard concentrations.

Determination of calcium concentrations

To determine calcium concentrations in serum, a commercial kit was utilized (Calcium oCPC, LT-CA0100, Labor+Technik Eberhard Lehmann GmbH, Germany) with some adaptations to allow for a higher throughput using a 96-well format instead of single cuvettes. For this, serum samples were first vortexed for homogenization before 2 µl per sample were transferred into a 96-well plate in duplicates. Similarly, 2 µl of the provided calcium standard was pipetted in duplicates. The cresolphthalein-complexon and an alkaline buffer, both included in the kit, were mixed 1:1 and 200 µl of the mix were added to each well. Wells containing neither a standard nor a sample were used as blank. After an incubation for 10 min at room temperature extinction was measured at 546 nm in a plate reader. Calcium concentrations were calculated based on the provided standard according to the manufacturers’ protocol.

Statistical analysis

In this study, we detected eight serum parameters, with cortisol additionally measured in saliva, in 15 mother sows housed in two different systems: a conventional ($n=7$) and an ecological facility ($n=8$). Additionally, phenotypic data of their litters were collected (Supplementary Table S1). Each pregnancy was considered an experimental unit, and data were collected across up to three pregnancies per sow, resulting in a total of 20 litters per husbandry system (Supplementary Table S3). For each parameter, we performed an outlier test (ROUT, $Q=1\%$), to curate the data before further statistical tests. Data processing and statistical analyses were conducted in R¹⁷ (version 4.4.2). A power analysis using the pwr package¹⁸ determined that a sample size of 20 per group could detect effect sizes of Cohen’s $d=0.9$ with a power of 0.80, classified as a large effect. To analyze the effects of housing and time on the detected neuroendocrine parameters, we fitted linear mixed-effects models (LMMs) using the lmerTest package¹⁹, which allows for hypothesis testing of fixed effects based on Satterthwaite’s degrees of freedom approximation. The response variables were log-transformed to meet model assumptions. The model included husbandry conditions (housing: conventional or eco), sampling time points (time: 105dpc or 8 dpd), and their interaction (housing \times time) as fixed effects.

To account for the hierarchical structure of the data, we included a random intercept for sows and an additional random intercept for litters nested within sows (1 | sow/litter). This structure reflects that each sow gave birth to up to three litters. The model was fitted using restricted maximum likelihood (REML), and parameter estimation was optimized using the bobyqa optimizer. To assess the significance of fixed effects, we conducted type II Wald chi-square tests using the car package²⁰. We also performed pairwise comparisons of housing conditions within each time point (and vice versa) using estimated marginal means (EMMs) via the emmeans package²¹. Pairwise contrasts were computed using Tukey’s adjustment for multiple comparisons to control for Type I error. The degrees of freedom were approximated using the Satterthwaite method and results were presented on the response scale. Standardized effect sizes (Cohen’s d) were calculated from the EMMs, accounting for uncertainty in both the estimated effects and the population standard deviation.

Principal component analysis and data imputation

Since the long-term ambition is to develop an objective biomarker for animal welfare, the application of unbiased methods such as unsupervised machine learning algorithms is an adequate method to test for the eligibility of candidate parameters. To investigate whether selected parameters could differentiate between the husbandry systems, we applied an unsupervised principal component analysis (PCA). For this, we condensed the data table to include only chosen parameters found to be deflected in dependence on the husbandry system or considered as the gold standard for stress detection (cortisol concentration). Since PCA requires complete cases, the analysis was initially limited to eight conventional and nine ecological litters, reducing sample size by over half and consequently lowering statistical power. Noteworthy, this reduction could also introduce bias.

To mitigate this issue, we applied an imputation method to replace missing values, ensuring that all cases were preserved. Notably, Missing values were imputed separately for each husbandry group to preserve group-specific characteristics. The K-Nearest Neighbors (KNN) imputer, accessible through the scikit-learn package

in Python²², was used to identify k-nearest neighbors for incomplete cases based on Euclidean distance that is suitable for continuous variables. Missing values were replaced with the mean of the nearest neighbors, applying uniform weighting. Neighbors adaptively adjusted when multiple missing values were present, ensuring context-sensitive imputation. To test for the feasibility of discriminating between the two husbandry systems based on the collected parameters, we conducted PCA using the mixOmics package²³ for both the original complete cases and the imputed dataset.

Results

The husbandry system does not affect quantity and weight of piglets but potentially the incidence of abnormalities

Given the involvement of the IGF system in growth and development, we examined the characteristics of litters born within our study cohort in the conventional and eco housing facilities. We could analyze 20 litters per husbandry system (Supplementary Table S1). During the study, sows gave birth to maximally three litters following inseminations at on average 310, 470 and 630 days of age for the standard husbandry and 270, 430 and 600 days for the ecological husbandry in our tested cohort. In both groups, data were collected relatively evenly across all parities ($n=7-8$ for 1st, $n=6-7$ for 2nd and $n=6$ for 3rd parity). The data revealed no significant differences in total litter size or sex distribution between conventional and ecological husbandry (Table 1). Also the proportion of low-birth-weight piglets (less than 1.1 kg at birth) was comparable between the two systems, with the most underweight piglets occurring in the first litters (conv: 56.3% (IQR 41.2–61.6), eco: 22.7% (IQR 11.8–37.9)), which is in line with data of other studies²⁴.

However, the incidence of litters with stillborn piglets was higher under conventional conditions. Furthermore, the occurrence of splay leg cases (piglets with defective leg muscles) was twice as high in the conventional system compared to the eco facility. To account for the potential confounding effects of the boars, we tested their influence on litter parameters. Except for the proportion of splay leg cases, boars had no significant impact. Only 9 out of 40 litters exhibited splay leg cases at all, with one boar showing relatively high occurrences (10.5% and 15.0% of piglets in a litter), leading to a significant confounding effect (Supplementary Table S4). However, this trait was not included in further analyses.

Cortisol levels in sows are similar in conventional and eco husbandry

Total cortisol concentrations were determined in the serum of pregnant and lactating sows across the two husbandry systems. As it represents a much less invasive approach, saliva cortisol concentrations are now more commonly analyzed to assess stress levels in pigs. Thus, we additionally detected cortisol in saliva. Since the housing conditions did not allow for controlling the hydration status of the sows, saliva cortisol concentrations were normalized to saliva protein content to account for potential dilution effects due to variations in salivary flow and drinking behavior. Of note, saliva sampling was generally performed before blood sampling.

For serum, cortisol concentrations did not differ significantly between husbandry systems or sampling time points (Table 2; Fig. 3A). Values ranged between 17.1 and 54.7 ng/ml. For saliva, slightly different results were obtained. Pregnant sows tended to have higher salivary cortisol concentrations in the conventional system compared to the eco husbandry (10.6 ng/mg (IQR 6.7–17.0) vs. 5.3 ng/mg (IQR 4.3–9.5)), while in lactating sows levels were similar in both husbandry systems (3.8 ng/mg (IQR 2.8–5.1) vs. 5.3 ng/mg (IQR 3.2–11.2)) as the values obtained of conventional husbandry have converged with those of the eco husbandry (Fig. 3B). Despite these numerical trends, none of the differences reached statistical significance (Table 2).

IGF bioactivity is affected by the housing environment

The proprietary BIRA assay was used to specifically detect IGF1R-mediated bioactivity, quantified based on AKT phosphorylation levels¹⁴. Since serum is a complex biological matrix potentially containing various compounds that could induce AKT phosphorylation, we verified the assay's specificity using the IGF1R inhibitor BMS-754,807. As expected, PBS with 50ng/ml IGF1 yielded a strong signal, while adding 0.1 μ M BMS reduced signal intensity to levels comparable to the negative control (PBS) (Supplementary Fig. S1, see also¹⁴). Similarly, in serum diluted 1:5 in PBS, the signal intensity dropped to negative control levels upon BMS supplementation, confirming that serum-induced AKT phosphorylation was specifically mediated by IGF1R. Even though IGF1 standards were utilized to assess IGF-dependent bioactivities, we provide data on IGF1R-mediated bioactivity as relative quantification to avoid potentially misleading concentration values (Fig. 3C).

The comparison of serum IGF bioactivities of differentially housed sows revealed significantly increased values under standard conditions (Table 2; Fig. 3C) in pregnant sows (86.9 (IQR 53.1–108.5) vs. 51.24 (IQR 35.4–80.7), $p=0.018$) and a trend to increased values under standard conditions in lactating sows (91.3 (IQR

Litter feature	Conventional ($n=20$)	Eco ($n=20$)
Total number of piglets per litter	17.9 \pm 3.1	16.7 \pm 3.9
Males per litter	50.1% \pm 13.7%	47.7% \pm 10.6%
Average birth weight	1.31 \pm 0.24 kg	1.37 \pm 0.23 kg
Low birth weight piglets per litter	29.8 \pm 24.9%	24.5 \pm 21.9%
Litters with still born	70.0%	50.0%
Litters with splay leg cases	30.0%	15.0%

Table 1. Comparison of basic features of litters depending on the husbandry system.

Factor	Chi-square	df	p-value
Cortisol (serum)			
Housing	0.041	1	0.840
Time	0.398	1	0.528
Housing×time	0.620	1	0.431
Cortisol (saliva)			
Housing	0.005	1	0.944
Time	0.897	1	0.344
Housing×time	2.546	1	0.111
IGF bioactivity			
Housing	5.456	1	0.020
Time	1.110	1	0.295
Housing×time	0.684	1	0.408
IGF1			
Housing	1.545	1	0.214
Time	37.204	1	<0.001
Housing×time	3.170	1	0.075
IGF2			
Housing	1.5551	1	0.212
Time	72.720	1	<0.001
Housing×time	8.298	1	0.004
IGFBP2			
Housing	6.451	1	0.011
Time	0.311	1	0.577
Housing×time	1.434	1	0.231
IGFBP3			
Housing	0.060	1	0.806
Time	23.658	1	<0.001
Housing×Time	0.030	1	0.863
IGFBP2/IGFBP3			
Housing	3.220	1	0.073
Time	26.410	1	<0.001
Housing×time	0.841	1	0.359
Proteolytic activity			
Housing	0.019	1	0.892
Time	0.001	1	0.974
Housing×time	0.416	1	0.519
STC1			
Housing	6.124	1	0.013
Time	12.473	1	<0.001
Housing×time	0.302	1	0.583
Calcium			
Housing	0.090	1	0.764
Time	0.533	1	0.465
Housing×time	1.536	1	0.215

Table 2. Analysis of deviance tables (type II Wald chisquare tests) of 10 analyzed parameters. For each factor (housing, time, and their interaction) chi-square values, degree of freedoms (df) and p values are given. Statistically significant factors are in bold.

67.6–118.2) vs. 70.5 (IQR 45.5–86.1), $p=0.109$). Calculated effect sizes for the husbandry conditions were 1.11 ± 0.46 at 105dpc, rendering this observation a large effect (Supplementary Table S5). In contrast, the time of sampling did not significantly affect IGF bioactivity, a finding further supported by the additional measurements at 30dpc (Supplementary Table S3).

Both IGF1 and IGF2 concentrations were measured in a subset of serum samples. At 105dpc, IGF1 levels showed a trend toward higher concentrations in conventional husbandry compared to the eco system (154.7 ng/ml (IQR 59.3–288.4) vs. 72.3 ng/ml (IQR 59.2–123.0), $p=0.064$); however, this difference was no longer evident at 8dpp (222.7 ng/ml (IQR 189.0–325.2) vs. 222.8 ng/ml (IQR 149.4–265.8), $p=0.779$). Since IGF2 also

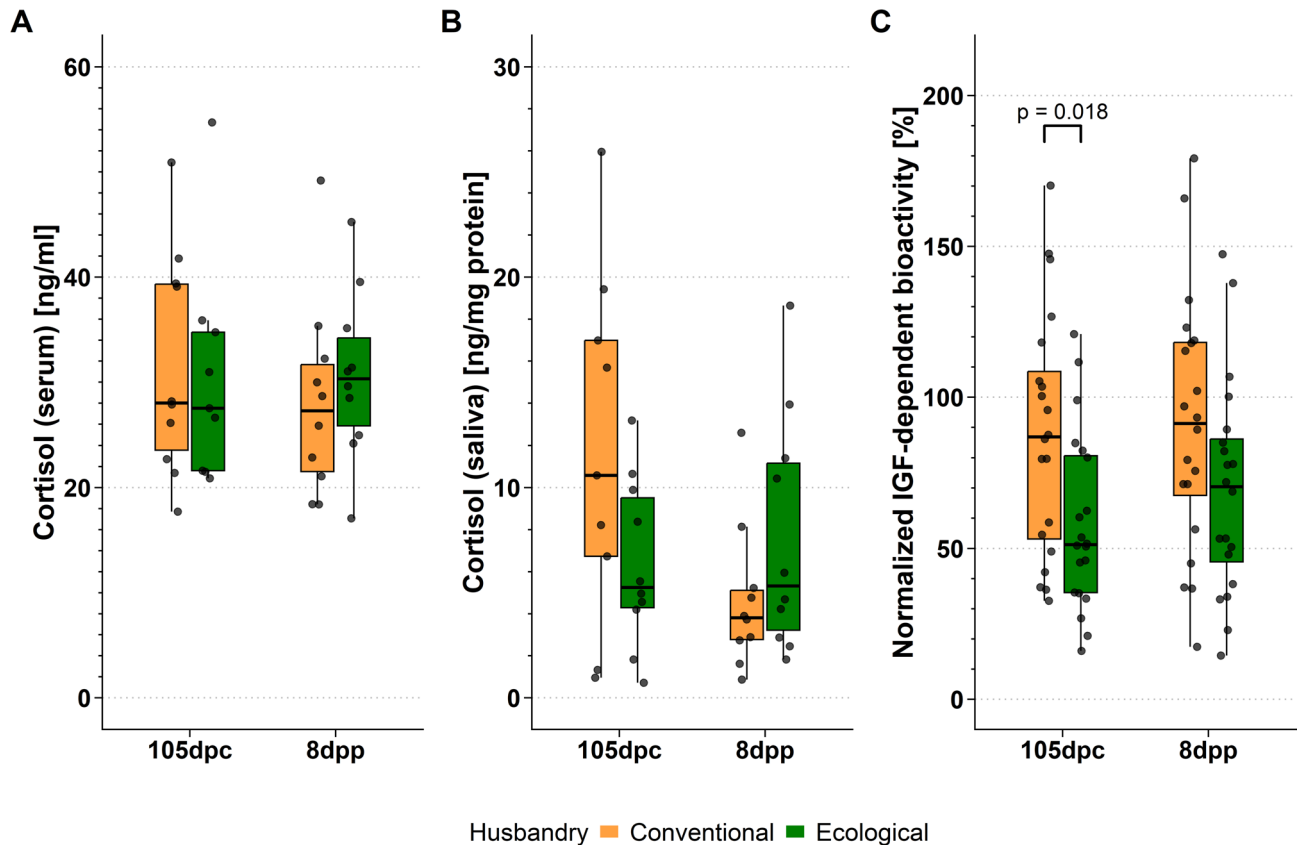


Fig. 3. Effects of the husbandry system on cortisol and IGF bioactivity. In differentially housed sows, cortisol concentrations were measured (A) in serum ($n=9-10$) and (B) in saliva, for which values were normalized to protein content to account for the varying water content ($n=9-10$). (C) Normalized IGF-dependent bioactivity was determined based on recombinant human IGF1 standards as reference ($n=20$). Statistical comparisons are based on estimated marginal means. Brackets indicate statistically significant differences with the corresponding p -values shown above.

activates IGF1R, thereby contributing to IGF-related bioactivity, IGF2 concentrations were measured in the same serum samples used for IGF1 analysis. Unlike IGF1, IGF2 levels were significantly elevated in conventional husbandry compared to eco husbandry at 105dpc (72.3 ng/ml (IQR 55.5–86.9) vs. 52.3 ng/ml (IQR 49.9–58.7), $p=0.008$), with an effect size of 1.38 ± 0.52 . At 8dpp, this difference was no longer observed (111.4 ng/ml (IQR 101.2–116.6) vs. 123.1 ng/ml (IQR 102.1–144.9), $p=0.381$). Both growth factors exhibited a significant increase in concentration from 105dpc to 8dpp in both housing systems (Table 2, $p<0.001$). However, this increase was more pronounced in sows housed in the eco facility, resulting in a convergence of the values at 8dpp (Fig. 4).

IGFBP2 but not IGFBP3 is affected by the housing environment

To address the question, if IGF-binding capacities, which control the activity of the IGFs, were affected by the husbandry system, Western ligand blotting was applied in the serum of our mother sow cohort. As expected IGFBP2 and IGFBP3 were the dominant binding proteins in serum, while IGFBP4 reached the detection level only in single replicates, which is why it was omitted for the comparative analysis.

For IGFBP2, housing conditions significantly influenced serum levels (Table 2, $p=0.011$). Similar to IGF1 and IGF2 concentrations this effect was more pronounced at 105dpc (conventional: 668.5 ng/ml (IQR 555.8–818.3) vs. ecological: 452.4 ng/ml (IQR 356.2–593.7), $p=0.013$), while at 8dpp IGFBP2 serum concentrations measured in sows in the eco facility were nearing those values observed for conventional husbandry (conventional: 593.3 ng/ml (IQR 501.3–784.0) vs. ecological: 573.9 ng/ml (IQR 325.3–619.1)) rendering the numerical difference no longer significant ($p=0.067$). Yet, the time of sampling was not found to have a statistically significant effect on serum IGFBP2 levels (Table 2, $p=0.577$).

In contrast, IGFBP3 levels were significantly increased in lactating sows compared to pregnant sows (Table 2, $p<0.001$), while housing conditions had no significant effect on this parameter (Table 2, $p=0.806$). In pregnant sows, IGFBP3 concentrations were 769.4 ng/ml (IQR 478.0–1572.0) under standard conditions and 840.8 ng/ml (IQR 533.9–1408.0) in eco husbandry. In lactating sows, concentrations were markedly higher, measuring 1573.6 ng/ml (IQR 751.8–3335.2) in conventional husbandry and 1954.0 ng/ml (630.1–3682.1) in eco husbandry. Due to this increase in IGFBP3 levels the IGFBP2/IGFBP3 ratio is significantly decreased in lactating sows (Fig. 5A).

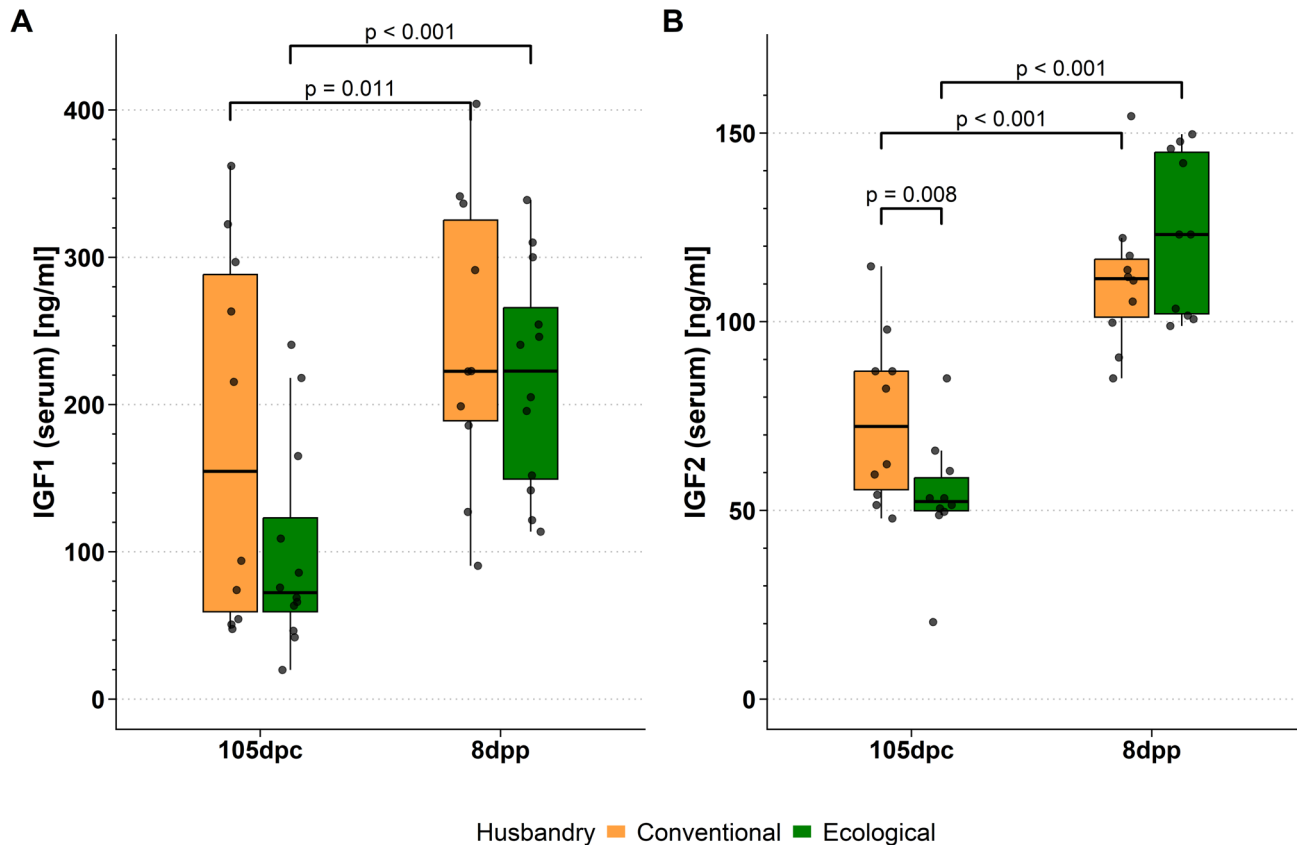


Fig. 4. Husbandry dependent IGF concentrations. Concentrations of total IGF1 (A) and IGF2 (B) were determined in serum of differentially housed pregnant and lactating sows ($n = 10$). Statistical comparisons are based on estimated marginal means. Brackets indicate statistically significant differences with the corresponding p-values shown above.

Proteolytic activity is minor in serum

Since IGF bioactivity also depends on the proteolytic activity of PAPP-A1/2, which cleaves binding proteins and thereby releases bioactive IGF, we also assessed proteolytic activity in the serum of the differentially housed sows. For this, we employed a Western ligand blot, detecting the amount of binding protein standards after increasing incubation times with the serum samples. Surprisingly, we observed extremely low proteolysis, which we then quantified by calculating the percentage decrease in detectable binding proteins after 24 h. Even after this extended incubation period, the observed values ranged only from 0% to 21%, with considerable variation between individuals and across different time points within the same individual (Supplementary Table S3). There were no significant differences based on housing conditions or between pregnant and lactating sows (Table 2). The mean values for all conditions fell within a narrow range of 6–7%. Estimates and standard errors of all measured parameters together with P-values for pairwise comparisons within time point and husbandry system are summarized in Supplementary Table S5.

STC1 levels are highly variable but overall lower under conventional husbandry conditions

As another crucial modulator of the overall IGF bioactivity, we investigated STC1 levels in serum of the differentially housed mother sows. For the conventional husbandry system, most measured values were below 200 pg/ml in both pregnant and lactating sows, with only a few samples exceeding 2000 pg/ml (Fig. 5B). A similar bimodal distribution was observed in sows from the eco husbandry system, but at a generally higher level. While some sows had STC1 concentrations below 2000 pg/ml, others exhibited values as high as 8000 pg/ml. Due to the unusual data distribution statistical models cannot be fitted simply. However, model diagnostics demonstrated a decent quality of a linear mixed model, especially regarding the normality of residues. Applying this model, sampling time point (pregnant vs. lactating) as well as husbandry form (ecological vs. conventional) were shown to demonstrate significant effects on STC1 concentrations (Fig. 5B and Supplemental Table S5).

Calcium levels are unaffected by the housing environment

Neither housing nor time point of sampling had a significant effect on calcium levels (Table 2), for which we measured values ranging from 2.5 to 26.5 mg/dl. Estimates and standard errors for both husbandry systems and measuring time points are presented in Supplementary Table S5.

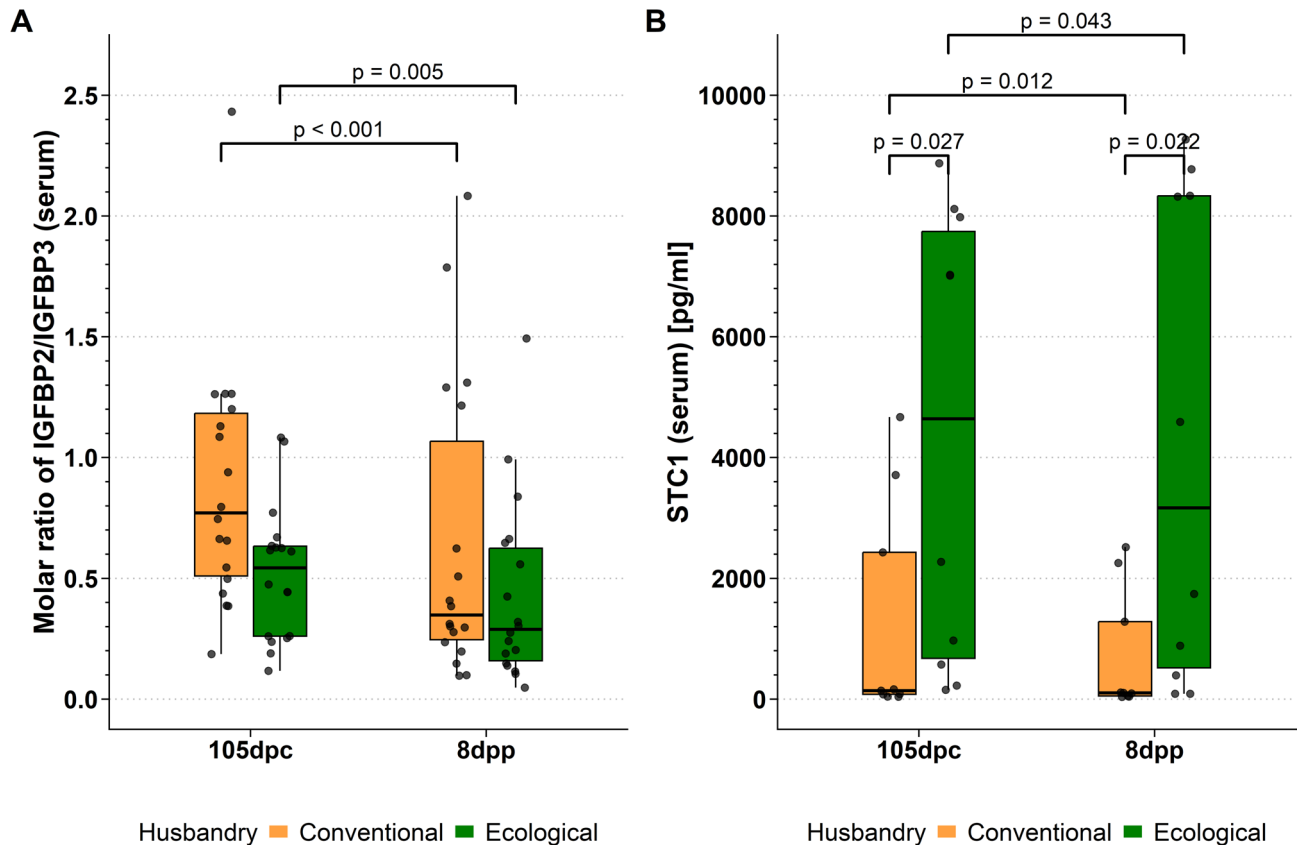


Fig. 5. Husbandry effects on regulators of IGF bioactivity (A) IGFBP2 concentrations were increased in conventional housing resulting in an altered IGFBP2/IGFBP3 ratio ($n = 18$). (B) STC1 concentrations in serum of pregnant and lactating sows ($n = 10$). Statistical comparisons are based on estimated marginal means. Brackets indicate statistically significant differences with the corresponding p-values shown above.

Factors of the IGF system partially distinguish husbandry systems

To identify underlying patterns and unsupervised groupings within the data, a PCA was performed based on 14 parameters, including serum IGF bioactivities, saliva cortisol concentrations, total IGF concentrations, binding protein concentrations (IGFBP2 and IGFBP3) and STC1 concentrations for 105 and 8dpp.

As PCA requires a complete dataset without missing values, the initial analysis was conducted on complete cases only, reducing the dataset to 10 cases from the conventional husbandry and 9 cases from the eco facility. While this analysis did not result in completely distinct data clusters, labeling the data points according to the husbandry system revealed that subgroups could be at least partially differentiated (Fig. 6A).

To minimize data loss and improve analytical accuracy, missing values were imputed using KNN imputation. This approach allowed for a more extensive dataset, increasing the sample size to 20 cases per husbandry system. PCA results for the imputed dataset reaffirmed the distinction between subgroups based on the candidate parameters (Fig. 6B). Importantly, variance patterns in the imputed dataset closely resembled those of the original non-imputed data, suggesting that the imputation algorithm preserved subgroup characteristics without introducing bias (Supplementary Fig. S2).

Analysis of feature weights for PC1 and PC2 indicated that serum IGF1 and STC1 concentrations, as well as IGFBP3 abundance accounted for the most variance in the reduced dataset (Fig. 6C). As expected, the principal components of the imputed dataset differed slightly from those of the reduced dataset due to the increased data input, reflected in differences in feature weights for PC1 and PC2 (Fig. 6D). Nevertheless, IGF1 and STC1 levels remained primary drivers of variation, while serum IGF bioactivities and IGFBP2 abundance also substantially contributed to differentiation between the two husbandry systems.

Discussion

Modulations of neuroendocrine factors are part of the animal's response to its environment. As such external conditions can affect the somatotrophic axis, which controls the IGF system. Notably, activity of the IGF system cannot be derived from isolated hormone concentrations, because a number of mechanisms are known to impact on bioavailability and bioactivity of the IGFs (Fig. 1)²⁵. In this study, we considered both, the overall IGF-related bioactivity and quantities of individual factors to investigate the impact of the housing environment, and welfare aspects in particular, on the downstream targets of the somatotrophic axis in farmed pigs. More generally, we aimed to clarify if the husbandry categories, defined by humans, represent distinct levels of welfare that can be

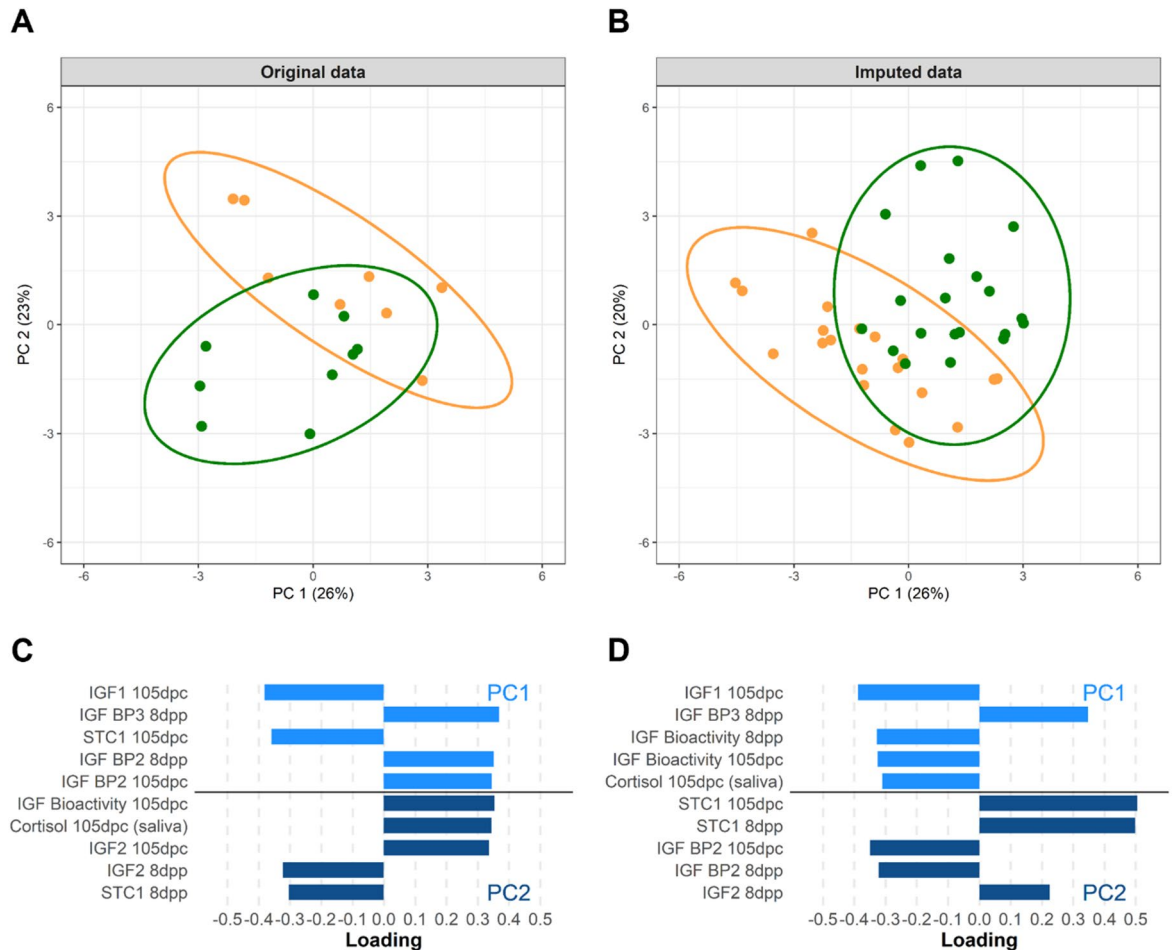


Fig. 6. PCA results for candidate parameters determined in the study (A) with a reduced dataset containing only complete cases and (B) with a greater dataset containing imputed data for missing values in the original dataset.

measured objectively. Indeed, we demonstrated a significantly increased serum IGF-dependent bioactivity under conventional husbandry conditions (German husbandry form 1), compared to the eco husbandry (German husbandry form 4/5) in pregnant sows. Moreover, we identified several individual factors of the IGF system to be significantly dependent on the husbandry system such as IGF2, IGFBP2 and STC1 (Table 2).

In contrast, the classical stress marker cortisol failed to reveal significant differences between the husbandry systems when single samplings were conducted at the measuring days. Even though samplings were conducted at similar time points for all animals, physiological fluctuations in cortisol secretion throughout the day and the seasons might contribute to the inconclusive results obtained for this hormone³. Similarly inconclusive results were obtained by Meunier-Salaun et al., who report lower cortisol levels in growing pigs both in groups with high and low area allocations compared to medium area allocations²⁶. Moreover, our results align with a study of Pearce and Paterson, who conducted repeated blood samplings in male pigs and demonstrated that basal cortisol concentrations are neither elevated by chronic space restriction nor reduced by access to toys²⁷. Intriguingly, growth rate was depressed under space restriction implying effects on the GH-IGF-axis.

However, another study in group-housed pregnant gilts by Barnett et al. reports higher plasma cortisol concentrations under space restriction²⁸. Of note, the offered space in this study from 1992 was generally extremely restricted compared to our study (0.98 m² or 1.97 m² per gilt vs. 4.0 m² or minimally 6.9 m² per gilt), which might explain these more pronounced differences in cortisol secretion. On the other hand, measured average cortisol concentrations were higher in our study (28.2–31.5 ng/ml) compared to the study of Barnett et al. (converted from nmol/l: 16.5–21.0 ng/ml), in which an implanted cannula was utilized for repeated measures. These elevated serum cortisol concentrations can potentially be attributed to the invasive blood sampling itself, which poses an acute stressor that affects cortisol secretion²⁹. For comparison, at 105 dpc normalized cortisol concentrations measured in saliva of the same sows ranged from 11.8 ± 8.4 ng/mg in conventional husbandry to 6.4 ± 4.0 ng/mg in eco husbandry, demonstrating a trend that is in accordance with the results of Barnett et al.

Overall, IGF bioactivities were not significantly affected by the measuring time point (pregnant vs. lactating sows), but concentrations of both IGF1 and IGF2 exhibited a significant increase in concentration from 105 dpc to 8 dpp in both husbandry systems. Increased IGF1 concentrations in lactating sows compared to pregnant

sows have been observed by others before^{30,31} and earlier results demonstrating increasing IGF1 and IGF2 serum concentrations throughout lactation³² imply a similar course. A study in rats suggests an effect of suckling on IGF1 release³³, which might be one aspect underlying the findings in our and other studies.

Interestingly, the increase in IGF1 and IGF2 concentrations is more pronounced in the eco facility, which results in diminished differences in serum concentrations between the husbandry systems at 8 dpp. A similar convergence was observed for IGFBP2 even though differences in IGFBP2 levels before and after farrowing did not reach statistical significance. In contrast, we found serum IGFBP3 levels to be significantly increased in lactating sows compared to pregnant sows. To our knowledge, there are no other studies investigating serum IGFBP3 concentrations in pregnant and lactating sows, but several lines of evidence suggest that IGFBP3 might act locally in brain regions responsible for maternal responsiveness and prolactin release following suckling³⁴.

Despite the presence of binding proteins in the serum, it might be noted that in some cases (4 out of 40) the IGF bioactivity, determined as IGF1-equivalents in ng/ml based on IGF1 standards, was higher than the sum of the measured total IGF1 and IGF2 concentrations. Several reasons can underly this non-intuitive result. First, due to its structural similarities, insulin can bind the IGF1R with low affinity and thereby activate the downstream signaling cascade, which can, to a limited extent, contribute to the final detected bioactivity^{35,36}. Of note, we did not analyze IGF1R levels in target tissues, as the mother sows were not killed for tissue sampling during this experiment. Second, even though IGFBPs are generally known as carriers that inhibit IGF bioactivity until its release, it is now known that IGFBP2 can enhance IGF1 signaling by vimentin mediated binding of receptor protein tyrosine phosphatase β , which eventually results in inhibiting the dephosphorylation of AKT by Phosphatase and Tensin homolog as demonstrated in vascular smooth muscle cells³⁷. We confirmed this promoting effect of IGFBP2 on IGF induced AKT phosphorylation in human embryonic kidney cells and tumorigenic epithelial-like cells¹⁴. In fact, we employ this effect to increase the sensitivity of our BIRA-Assay. In view of this, the elevated IGFBP2 abundance that we observed in the conventional husbandry might contribute to the increased IGF bioactivity that was demonstrated here.

For another crucial modulator of the overall IGF bioactivity, namely STC1, levels seemed to be decreased under standard conditions both in pregnant and lactating sows. Since STC1 inhibits the proteolytic activity of PAPP-A, thereby limiting the release of IGF from its binding proteins (Fig. 1)¹², this is in line with the increased bioactivity that was observed under standard conditions. However, surprisingly, the proteolytic activity was extremely low in all samples and did not reveal any significant differences between the husbandry systems. Accordingly, there might be another mechanism by which differences in STC1 concentrations translate into differential bioactivities. Literature on the linkages between STC1 and AKT phosphorylation (readout of our bioactivity assay) are inconsistent. While a study by Yang et al. reports that STC1 promotes the phosphorylation of AKT³⁸, a recent study of Kim et al. states that STC1 strongly inhibited AKT phosphorylation³⁹. Further studies imply that STC1 expression itself is positively⁴⁰ or negatively⁴¹ regulated by AKT phosphorylation. Apparently, the function and regulation of STC1 is highly tissue and species dependent, rendering further studies in pig crucial to elucidate the relevance of differential serum STC1 concentrations. However, studies involving mammalian STC1 are complicated by the fact that circulating serum STC1 is rapidly eliminated⁴². In fact, STC1 expression predominantly occurs in reproductive tissues of pregnant females, while circulating STC1 is reported to be hardly detectable in nonpregnant individuals⁴³. For mice before and after parturition maximum STC1 levels of 1,600 pg/ml were reported⁴⁴, while for cows values ranged between 5,000 and 8,000 pg/ml⁴⁵. Mean values of 8,400 pg/ml were detected in porcine follicular fluids⁴⁶, which is similar to the peak values measured in serum of our mother sow cohort.

While we identified several individual factors of the IGF system to be dependent on the husbandry system (Table 2; Fig. 7), there are still some limitations to their applicability as biomarkers. For example, STC1 concentrations demonstrated very high inter-individual variances, while effects of the husbandry system on IGF bioactivity as well as IGF2, and IGFBP2 levels were only significant in pregnant but not in lactating sows. Nevertheless, our unsupervised approach suggests that factors of the IGF system can be potentially utilized to identify groups based on their housing environment.

The imputation of missing data by bioinformatics methods can further support data analysis by unsupervised approaches. Among available methods, we selected KNN imputation, which is particularly well-suited for small datasets with continuous data and complex biological variation. By this means, we identified STC1 levels and IGF1 concentration as the main contributors to the observed variance in our dataset. This leads us to the conclusion that the IGF system might be well suited to assess differences in general welfare, even if the development of a suitable biomarker could still be a long way off. Moreover, our results imply that husbandry categories for pigs indeed represent distinct levels of welfare that can be measured objectively. However, it needs to be noted that we compared husbandry form 1 and 4, while we believe that we would not have obtained similarly clear results for the comparisons of husbandry form 1 vs. 2 or husbandry form 3 vs. 4.

Surprisingly, IGFBP3 abundance contributed significantly to the principal components that allowed for a distinction of the husbandry systems, despite the fact that differences in IGFBP3 concentrations between the husbandry systems were not found to be statistically significant neither for the reduced nor for the full dataset. Similarly, serum IGF 1 and saliva cortisol concentrations at 105dpc were involved in the observed group separation while not demonstrating statistically significant differences between the two groups. This underpins the notion that measuring individual molecule concentrations might not be sufficient to assess chronic stress and distinguish between husbandry forms. While considering the interplay of multiple factors is one approach to tackle this issue^{8,9}, we showcased here that detecting downstream signaling might be another well suited approach. Ultimately, however, only a multifactorial and integrative approach is likely to provide the necessary robustness for the development of biomarkers to objectively evaluate animal welfare.

Concerning the physiological relevance of our findings, a number of studies suggest that deflections of the IGF system are not only a by-product of stress responses that can be exploited for biomarker development but

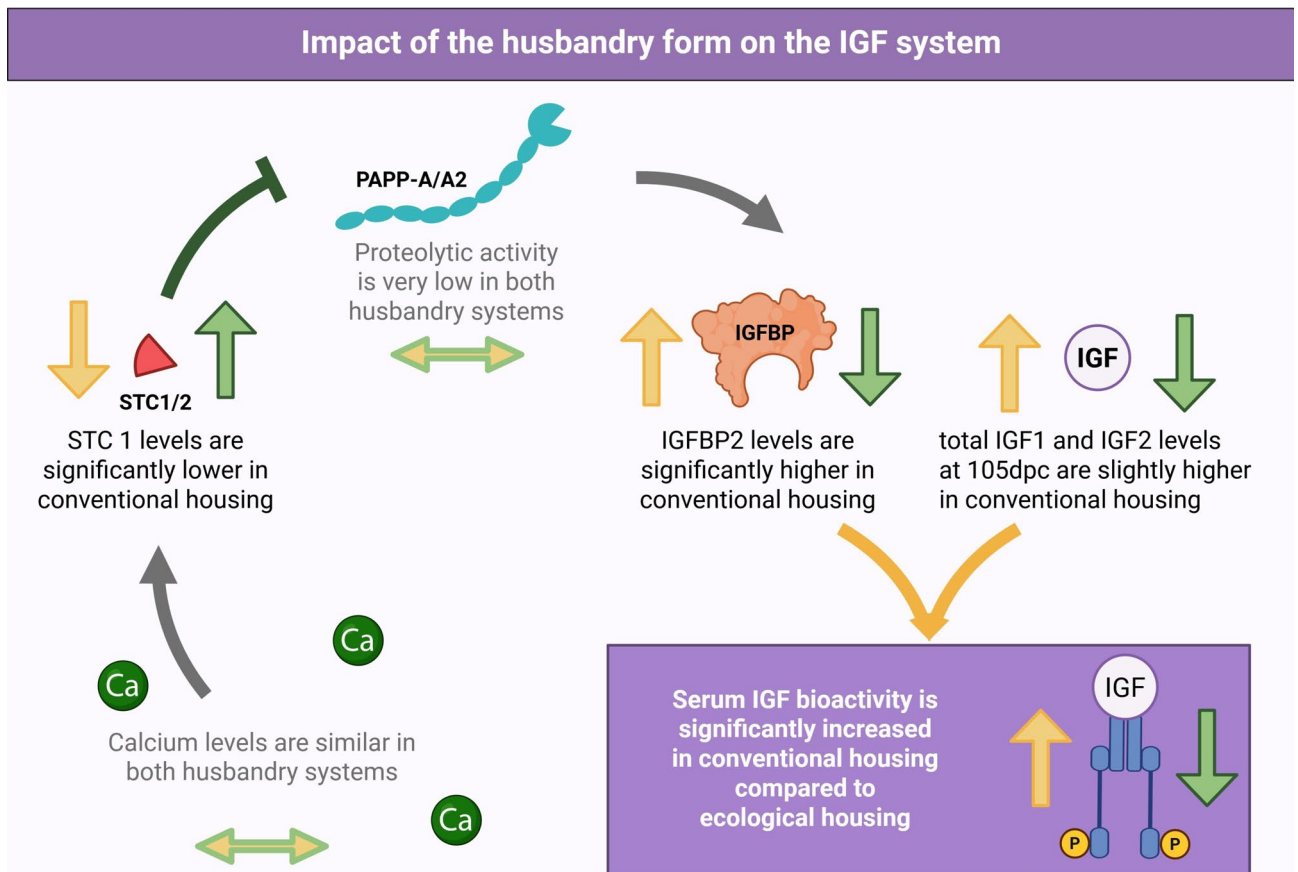


Fig. 7. Overview on the differential impact of the husbandry system on the abundance and bioactivity of factors of the IGF system.

that the system functionally contributes to the animals' stress coping capacity. Findings across different species indicate that circulating IGF1 is involved in mood homeostasis^{47,48}. For example, IGF1 administration was repeatedly shown to initiate cascades of neurochemical effects resulting in antidepressant activity as reviewed by Szczyński et al.⁴⁹. Similar effects are observed under treatment with the nonspecific IGF1 inhibitor NBI-31,772⁵⁰. Suggested underlying mechanisms include the promotion of other neurotrophic factors such as Brain derived neurotrophic factor⁵¹, upregulation of serotonin levels in the hippocampus⁵², sensitivity regulation of neuronal glucocorticoid receptors⁴⁷, and modification of GABA/Glutamate synaptic structures in orexin neurons⁴⁸. Accordingly, the increased IGF1 bioactivity that we found in conventional husbandry might reflect the sows' compensative answer to the more stressful environment.

Moreover, it was reported that overexpression of mutant IGFBP2 reduced anxiety in mice indicating central functions of the IGF system⁵³. Intriguingly, we found increased serum IGFBP2 levels in sows housed under conventional husbandry conditions, which is also in line with the hypothesis that factors of the IGF system play a relevant role for stress coping mechanisms.

It needs to be mentioned that the fact that the test animals were only sampled alive and not slaughtered during our longitudinal study leads to a certain limitation. While we monitored all relevant parameters upstream of the IGF1R as well as downstream bioactivity, we could not detect IGF1R expression levels in target tissues. Therefore, we could not assess any regulatory mechanisms on the level of the IGF1 receptor, which are known to be crucial for the translation of signals into pleiotropic biological functions⁵⁴, while differential sensitivity of receptor variants can even add another layer of complexity⁵⁵. For the same reason, it was not possible to detect STC1 in reproductive tissues of investigated sows.

Another potential limitation was introduced by the situation that successfully inseminated sows happened to be younger on average in the ecological facility. This led us to analyze the potential of age as confounding factor. In fact, we observed a significant effect of the animals age on serum concentrations of IGF1, IGFBP2 and STC1, which might imply a habituation process in the sows. Nevertheless, IGF bioactivity under eco conditions was found to be generally lower and these effects were extremely small (Supplementary Table S4). Moreover, this effect is unlikely to be relevant in industrial pig farming, as the time to slaughter is much shorter than the lifetime of the sows examined here. However, further studies will be necessary to assess the compliance of the German husbandry form 4/5 with the animals needs and to elucidate options to continuously improve animal welfare in agriculture.

Conclusions

Our results imply that factors of the somatotrophic axis might provide a solid basis for the documentation and objective assessment of animal health and welfare. Potentially, alterations in the IGF system might even indicate illness or chronic stress before symptoms manifest themselves externally in the animal thus allowing for early interventions. However, while we identified several individual factors of the IGF system to be dependent on the husbandry system, there are limitations to their applicability as biomarkers such as high inter-individual variances and changing effect sizes. Ultimately, only a multifactorial and integrative approach is likely to provide the necessary robustness for developing biomarkers to objectively evaluate animal welfare.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets and R code used for statistical analysis and visualization in this study are available on GitHub at <https://github.com/AnjaEggert/IGF-animal-welfare.git>.

Received: 3 December 2025; Accepted: 25 February 2026

Published online: 04 March 2026

References

1. BMLEH - Publications. *Future-proof animal husbandry - Cornerstones for the introduction of mandatory state animal husbandry labelling*. https://www.bmleh.de/SharedDocs/Downloads/EN/_Animals/key-points-animal-husbandry.html (2022).
2. Dalmau, A. et al. *Welfare Quality' Assessment for Pigs (Sows and Piglets, Growing and Finishing Pigs)*. (2009).
3. Ruis, M. A. W. et al. The circadian rhythm of salivary cortisol in growing pigs: Effects of age, gender, and stress. *Physiol. Behav.* **62**, 623–630 (1997).
4. Mormède, P. et al. Exploration of the hypothalamic–pituitary–adrenal function as a tool to evaluate animal welfare. *Physiol. Behav.* **92**, 317–339 (2007).
5. Jensen, K. H. et al. Intermittent stress in pigs: Effects on behavior, pituitary — Adrenocortical axis, growth, and gastric ulceration. *Physiol. Behav.* **59**, 741–748 (1996).
6. Wiechers, D. H., Brunner, S., Herbrandt, S., Kemper, N. & Fels, M. Analysis of Hair Cortisol as an Indicator of Chronic Stress in Pigs in Two Different Farrowing Systems. *Front. Vet. Sci.* **8**, 605078 (2021).
7. Wirthgen, E. et al. Interference of stress with the somatotrophic axis in pigs – lights on new biomarkers. *Sci. Rep.* **7**, 12055 (2017).
8. Wirthgen, E. et al. Interference of stress with the somatotrophic axis in pigs – lights on new biomarkers. *Sci. Rep.* **7**, 12055 (2017).
9. Wirthgen, E. et al. Effects of Transport Duration and Environmental Conditions in Winter or Summer on the Concentrations of Insulin-Like Growth Factors and Insulin-Like Growth Factor-Binding Proteins in the Plasma of Market-Weight Pigs. *Front. Endocrinol. (Lausanne)*. **9**, 36 (2018).
10. Allard, J. B. & Duan, C. IGF-Binding Proteins: Why Do They Exist and Why Are There So Many? *Front. Endocrinol. (Lausanne)*. **9**, 117 (2018).
11. Oxvig, C. The role of PAPP-A in the IGF system: Location, location, location. *J. Cell. Communication Signal.* **9**, 177 (2015).
12. Kløverpris, S. et al. Stanniocalcin-1 potently inhibits the proteolytic activity of the metalloproteinase pregnancy-associated plasma protein-A. *J. Biol. Chem.* **290**, 21915–21924 (2015).
13. Dai, W. et al. Calcium deficiency-induced and TRP channel-regulated IGF1R-PI3K-Akt signaling regulates abnormal epithelial cell proliferation. *Cell Death Differ.* **21**, 568–581 (2014).
14. Walz, M. et al. Development of a sensitive bioassay for the analysis of IGF-related activation of AKT/mTOR signaling in biological matrices. *Cells* **10**, 482 (2021).
15. Flachowsky, G. et al. Empfehlungen zur Energie- und Nährstoffversorgung von Schweinen 2006. *Energie- und Nährstoffbedarf landwirtschaftlicher Nutztiere*. **10**, 247 (2006).
16. Höflich, A., Lahm, H., Blum, W., Kolb, H. & Wolf, E. Insulin-like growth factor-binding protein-2 inhibits proliferation of human embryonic kidney fibroblasts and of IGF-responsive colon carcinoma cell lines. *FEBS Lett.* **434**, 329–334 (1998).
17. R: *The R Project for Statistical Computing*. <https://www.r-project.org/>.
18. Champely, S. *pwr: Basic Functions for Power Analysis*. 1.3–0 <https://doi.org/10.32614/CRAN.package.pwr> (2006).
19. Kuznetsova, A. & Bruun Brockhoff, P. & Haubo Bojesen Christensen, R. *lmerTest: Tests in Linear Mixed Effects Models*. 3.1-3 <https://doi.org/10.32614/CRAN.package.lmerTest> (2013).
20. Fox, J., Weisberg, S. & Price, B. *car: Companion to Applied Regression*. 3.1-3 <https://doi.org/10.32614/CRAN.package.car> (2001).
21. Lenth, R. V. & Emmeans *Estimated Marginal Means, aka Least-Squares Means*. 1.10.7 <https://doi.org/10.32614/CRAN.package.emmeans> (2017).
22. Pedregosa, F. et al. Scikit-learn: Machine Learning in Python. *J. Mach. Learn. Res.* **12**, 2825–2830 (2011).
23. Rohart, F., Gautier, B., Singh, A. & Cao, K.-A. mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Comput. Biol.* **13**, e1005752 (2017).
24. Langendijk, P., Fleuren, M. & Page, G. Review: Targeted nutrition in gestating sows: opportunities to enhance sow performance and piglet vitality. *Animal* **17**, 100756 (2023).
25. Argente, J., Chowen, J. A., Pérez-Jurado, L. A., Frystyk, J. & Oxvig, C. One level up: Abnormal proteolytic regulation of IGF activity plays a role in human pathophysiology. *EMBO. Mol. Med.* **9**, 1338–1345 (2017).
26. Meunier-Salaun, M. C., Vantrimponte, M. N., Raab, A. & Dantzer, R. Effect of floor area restriction upon performance, behavior and physiology of growing-finishing pigs. *J. Anim. Sci.* **64**, 1371–1377 (1987).
27. Pearce, G. & Paterson, A. M. The effect of space restriction and provision of toys during rearing on the behaviour, productivity and physiology of male pigs. *Appl. Anim. Behav. Sci. - APPL. ANIM. BEHAV. SCI.* **36**, 11–28 (1993).
28. Barnett, J. L. et al. Effects of pen size, partial stalls and method of feeding on welfare-related behavioural and physiological responses of group-housed pigs. *Appl. Anim. Behav. Sci.* **34**, 207–220 (1992).
29. Merlot, E., Mounier, A. M. & Prunier, A. Endocrine response of gilts to various common stressors: A comparison of indicators and methods of analysis. *Physiol. Behav.* **102**, 259–265 (2011).
30. Schams, D., Kraetzl, W. D., Brem, G. & Graf, F. Secretory pattern of metabolic hormones in the lactating sow. *Exp. Clin. Endocrinol. Diabetes.* **102**, 439–447 (1994).
31. Lee, C. Y., Baik, K. H., Lee, D. H. & Park, H. C. Relationships of plasma insulin-like growth factor (IGF)-I and IGF-II concentrations to litter size. *J. Anim. Sci. Technol.* **45**, 33–40 (2003).
32. Donovan, S. M., Mcneil, L. K., Jiménez-flores, R. & Odle, J. Insulin-like growth factors and insulin-like growth factor binding proteins in porcine serum and milk throughout lactation. *Pediatr. Res.* **36**, 159–168 (1994).
33. Lékó, A. H., Cservenák, M. & Dobolyi, A. Suckling induced insulin-like growth factor-1 (IGF-1) release in mother rats. *Growth Horm. IGF Res.* **37**, 7–12 (2017).

34. Dobolyi, A. & Lékó, A. H. The insulin-like growth factor-1 system in the adult mammalian brain and its implications in central maternal adaptation. *Front. Neuroendocr.* **52**, 181–194 (2019).
35. Rinderknecht, E. & Humbel, R. E. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J. Biol. Chem.* **253**, 2769–2776 (1978).
36. Varewijck, A. J. & Janssen, J. A. M. J. L. Insulin and its analogues and their affinities for the IGF1 receptor. *Endocr. Relat. Cancer* **19**, F63–F75 (2012).
37. Shen, X. et al. Insulin-like growth factor (IGF) binding protein 2 functions coordinately with receptor protein tyrosine phosphatase β and the IGF-I receptor to regulate IGF-I-stimulated signaling. *Mol. Cell. Biol.* **32**, 4116–4130 (2012).
38. Yang, Y., Yin, S., Li, S., Chen, Y. & Yang, L. Stanniocalcin 1 in tumor microenvironment promotes metastasis of ovarian cancer. *Oncol. Targets Ther.* **12**, 2789–2798 (2019).
39. Kim, J. H. et al. Stanniocalcin 1 and 1,25-dihydroxyvitamin D3 cooperatively regulate bone mineralization by osteoblasts. *Exp. Mol. Med.* **56**, 1991–2001 (2024).
40. Jeon, M., Han, J., Nam, S. J., Lee, J. E. & Kim, S. STC-1 expression is upregulated through an Akt/NF- κ B-dependent pathway in triple-negative breast cancer cells. *Oncol. Rep.* **36**, 1717–1722 (2016).
41. Yeung, B. H. Y. & Wong, C. K. C. Stanniocalcin-1 Regulates Re-Epithelialization in Human Keratinocytes. *PLoS One.* **6**, e27094 (2011).
42. De Niu, P. et al. Development of a human stanniocalcin radioimmunoassay: Serum and tissue hormone levels and pharmacokinetics in the rat. *Mol. Cell. Endocrinol.* **162**, 131–144 (2000).
43. Bishop, A., Cartwright, J. E. & Whitley, G. S. Stanniocalcin-1 in the female reproductive system and pregnancy. *Hum. Reprod. Update.* **27**, 1098–1114 (2021).
44. Deol, H. K., Varghese, R., Wagner, G. F. & DiMattia, G. E. Dynamic regulation of mouse ovarian Stanniocalcin expression during gestation and lactation*. *Endocrinology* **141**, 3412–3421 (2000).
45. Tremblay, G. et al. Serum levels of stanniocalcin-1 in Holstein heifers and cows. *Domest. Anim. Endocrinol.* **36**, 105–109 (2009).
46. Basini, G. et al. Expression and localization of stanniocalcin 1 in swine ovary. *Gen. Comp. Endocrinol.* **166**, 404–408 (2010).
47. Santi, A., Bot, M., Aleman, A., Penninx, B. W. J. H. & Aleman, I. T. Circulating insulin-like growth factor I modulates mood and is a biomarker of vulnerability to stress: From mouse to man. *Transl. Psychiatry.* **8**, 1–11 (2018).
48. Fernández de Sevilla, M. E. et al. Insulin-like growth factor I mitigates post-traumatic stress by inhibiting AMP-kinase in orexin neurons. *Mol. Psychiatry.* **27**, 2182–2196 (2022).
49. Szczygny, E. et al. Possible contribution of IGF-1 to depressive disorder. *Pharmacol. Rep.* **65**, 1622–1631 (2013).
50. Malberg, J. E. et al. Increasing the levels of insulin-like growth factor-I by an IGF binding protein inhibitor produces anxiolytic and antidepressant-like effects. *Neuropsychopharmacology* **32**, 2360–2368 (2007).
51. McCusker, R. H. et al. Insulin-like growth factor-I enhances the biological activity of brain-derived neurotrophic factor on cerebrocortical neurons. *J. Neuroimmunol.* **179**, 186–190 (2006).
52. Hoshaw, B. A. et al. Antidepressant-like behavioral effects of IGF-I produced by enhanced serotonin transmission. *Eur. J. Pharmacol.* **594**, 109–116 (2008).
53. Schindler, N. et al. Phenotype analysis of male transgenic mice overexpressing mutant IGFBP-2 lacking the Cardin-Weintraub sequence motif: Reduced expression of synaptic markers and myelin basic protein in the brain and a lower degree of anxiety-like behaviour. *Growth Horm. IGF Res.* **33**, 1–8 (2017).
54. Choi, E., Duan, C. & Bai, X. Regulation and function of insulin-IGF receptor signaling. *Nat. Rev. Mol. Cell. Biol.* **26**, 558–580 (2025).
55. Muráni, E. et al. A naturally hypersensitive glucocorticoid receptor elicits a compensatory reduction of hypothalamus–pituitary–adrenal axis activity early in ontogeny. *Open. Biol.* **6**, 150193 (2016).

Acknowledgements

We want to acknowledge all people involved in the animal experiment for their continuous support from the submitting of the animal experiment application to the daily care of the animals. Moreover, we would like to thank Luong Chau for her efforts in processing the numerous SDS gels and Sebastian Galuska for providing support for figure generation. Figures 1, 2 and 7 were generated in BioRender.

Author contributions

Phenotypic parameters of mother sows and piglets were recorded by MZ, who was involved in the organization of the animal experiment. AG and DO were involved in the sampling and processing of biological matrices as well as subsequent molecular biological experiments. CW performed the calcium measurements. CU and SB performed the data imputation and PCA under supervision of OW, who together with SB also served as a general consultant for the bioinformatic analysis. Hypothesis formulation and study conceptualization was provided by AH. Statistical analyses and visualizations were executed by AE and AG. Project management, manuscript conceptualization and writing was performed by AG, while text editing was carried out by AE, CU, and AH. CW and OW contributed to the final revision. All authors read and approved the final manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-026-42315-3>.

Correspondence and requests for materials should be addressed to A.-M.G. or A.H.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2026