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Caroline Clouard, Rémi Resmond, Héloïse Vesque-Annear, Armelle Prunier, Elodie Merlot

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Pre-weaning social behaviours and peripheral serotonin levels are associated with behavioural and physiological responses to weaning and social mixing in pigs

Caroline Clouard *, Rémi Resmond, Héloïse Vesque-Annear, Armelle Prunier, Elodie Merlot

PEGASE, INRAE, Institut Agro, 35590 Saint-Gilles, France

* Corresponding author.

E-mail address: caroline.clouard@inrae.fr (C. Clouard)

ABSTRACT

In pig production systems, weaning is a major challenge that is usually paired with social mixing and may greatly affect health and welfare of piglets. Research efforts have been devoted to characterising early predictors of weaning adaptation, but have focused mainly on aggressive and harmful behaviours, whereas socio-positive behaviours have been poorly studied. Furthermore, serotonin (5-HT), a neurotransmitter regulating social behaviours, may also be a pertinent predictor of piglets' adaptation to challenging situations. This study aimed to assess whether social behaviours and blood 5-HT concentration before weaning were associated with behavioural and physiological responses of piglets to weaning. Social interactions (social exploration, aggression, play-fight, locomotor play) of 72 focal piglets from 12 litters were scored continuously for 8h at 42 days of age. At weaning (d48), focal piglets were allocated to four pens of 33 piglets from six litters. During the two days following weaning (d49-50), social interactions were scored continuously for 6h per day, and behavioural activities were scored with 6-min instantaneous scan sampling. Blood was sampled one week before (d41) and 24h after (d49) weaning to measure 5-HT concentrations and health-related variables. Exploration of pen mates represented 55% and 79% of all scored social interactions before and after weaning, respectively, and play was not observed after weaning. Using a multivariate analysis paired with clustering analysis on post-weaning behavioural and physiological responses, we identified three clusters of piglets with distinct profiles of adaption to weaning: *unhealthy inactive animals*, *healthy inactive aggressors* and *healthy active affiliative animals*. Compared to other clusters, *unhealthy inactive animals* at weaning were characterised by lower levels of social exploration and aggression before weaning ($p < 0.05$ for both). Furthermore, piglets that explored their pen mates more before weaning were more active ($p = 0.03$) after weaning, while piglets that were involved in greater number of locomotor play episodes ($p = 0.009$) or that were less aggressive ($p = 0.04$) before weaning walked more after weaning. Piglets with higher blood 5-HT concentrations before weaning were less aggressive ($p = 0.01$) and had greater growth ($p = 0.009$) after weaning. Pre-weaning aggression was also positively associated with post-weaning lymphocyte count ($p = 0.04$), and pre-weaning locomotor play with post-weaning hydroperoxide concentration ($p = 0.05$), a marker of oxidative stress. Our findings suggest that pre-weaning social behaviours and blood 5-HT concentration may be relevant predictors of piglets' adaptive responses to social mixing at weaning and deserve more research attention.

Keywords: Social nosing; Play behaviour; Aggression; Adaptation; Welfare; Piglets

1. Introduction

The pig is a social animal that exhibits a variety of social behaviours. In stable social groups, except in situations where competition for limited resources is high due to specific housing or feeding conditions, agonistic behaviours are relatively rare, and pigs exhibit predominantly non-agonistic social behaviours, such as social nosing (Beattie et al., 2000; Clouard et al., 2022). Social nosing, or social exploration, is typically assumed to contribute to both individual recognition and affiliation and to play a role in social cohesion (Camerlink and Turner, 2013). Social play, a behaviour exhibited predominantly by immature animals, is another non-agonistic social behaviour that is assumed to help piglets acquire motor and social skills, needed for successful fighting in adulthood (Horback, 2014). However, in farms, mixing of individuals at various stages of the production process often challenges the social organization, generating welfare issues.

In pig production systems, the transition from milk to solid feed at weaning is typically associated with changes in the physical environment, with piglets being moved to an unfamiliar pen or barn. Weaning is also considered a major social challenge, with piglets being separated from the sow and from at least some of their littermates, while being regrouped with piglets from other litters (Weary et al., 2008). Mixing with unfamiliar animals usually results in vigorous and intense fights, which are needed to establish a new social hierarchy (Meese and Ewbank, 1973). In addition, newly-weaned piglets often have difficulty initiating feeding (Metz and Gonyou, 1990). Weaning is thus associated with behavioural responses that are assumed to reflect the piglet's decline in well-being. Early-weaned piglets often show abnormal damaging social behaviours, such as belly nosing (Widowski et al., 2008). Furthermore, a profound depression of play is typically observed (Donaldson et al., 2002). Piglets weaned early also explore their environment less (nosing and chewing objects; Worobec et al., 1999) and spend more time lying or resting directly after weaning (Metz and Gonyou, 1990). These behavioural alterations are often accompanied by physiological changes reflective of degraded health, such as a rise in blood markers of oxidative stress (hydroperoxides; Sauerwein et al., 2007; Buchet et al., 2017) and inflammation (haptogoblin; Sauerwein et al., 2007), especially in early-weaned piglets. A better understanding of how early (social) behaviour and health status shape the pig coping responses when facing a social challenge is important to optimize rearing practices and limit social stress.

Research efforts have been made to identify predictors or markers of piglets' responses to social challenges. For instance, agonistic behaviour after mixing has been found to be predicted by early behavioural traits, such as aggressiveness, responses towards humans, and coping style (reactive vs proactive; Erhard et al., 1997; Melotti et al., 2011; Scheffler et al., 2016). These traits, however, were assessed in a variety of behavioural tests performed outside of the living pen and little is known on the association between pre-weaning home pen behaviours, which are easier to assess on-farm, and behavioural responses to weaning and social mixing. Recently, we highlighted the existence of different social styles in immature suckling piglets (Clouard et al., 2022), which can be evaluated in the home-pen, and suggested the existence of different sociality traits, including ‘sociability’, ‘aggressiveness’ and ‘avoidance’. In light of these findings, we hypothesize that early social behaviour and style may partly determine how the animal will respond to social challenges later in life. In addition to early behavioural traits, the characterisation of physiological markers of sociality may facilitate the identification of animals with a low capacity to adapt to social challenges. The brain serotonin (5-HT) or its metabolites have been positively correlated to affiliative social behaviour (grooming) and negatively to aggression in human and non-human primates (Insel and Winslow, 1998). In pigs, reduced blood 5-HT concentrations have been associated with high levels of aggression (Poletto et al., 2010) and tail biting (Ursinus et al., 2014), suggesting that peripheral 5-HT may represent a valid proxy to cerebral serotoninergic activity and be a pertinent biomarker of social responses to weaning. Finally, piglets are immature animals that frequently, but usually transiently, experience various health problems, *e.g*. digestive problems. These problems, by generating an immune and inflammatory response, are likely to induce sickness behavioural responses.

Therefore, this study aimed to (1) characterise distinctive profiles of adaption to weaning and social mixing in piglets based on behavioural responses to weaning and physiological indicators of health, and (2) determine whether social variables (social interactions and peripheral 5-HT concentrations) prior to weaning were associated with these adaptive responses to weaning. To achieve these aims, we used pigs that were involved in a larger project that aimed to test methods of early iron supplementation in suckling piglets raised in organic farming, and in which the housing conditions (low density, enriched housing, free farrowing pens) offer optimal conditions to study social behaviour in young pigs.

2. Material and methods

The experiment was conducted at the French National Institute INRAE organic pig farm Porganic (Rouillé, France) from May to October 2020 in compliance with the current ethical standards of the European Community (Directive 2010/63/EU). The experimental procedures used in this study were approved by the Regional Ethics Committee in Animal Experiments of Poitou Charente (n°084, December 18, 2019) and by the French Ministry of Higher Education and Research (2019071611422718Apafis21892).

2.1. Animals and housing

Piglets (Large White × Piétrain) from 12 litters (14.5 ± 0.48 [range: 12-18] piglets born alive per litter; male:female ratio = 1.6) were studied in two consecutive cohorts, with 6-week intervals between cohorts and six litters per cohort. From birth until 48 days of age, all piglets were kept with the sows that were locked in farrowing crates from entry to the farrowing pens (at 105 days of gestation) until four days after farrowing, and loose thereafter. All sows and their piglets were housed in 10-m^2 indoor individual farrowing pens on straw bedding, located within the same maternity building, until 11 days after farrowing. Then, sows and their piglets were moved to a neighbouring maternity unit and housed in individual farrowing pens consisting of a 10-m^2 indoor area on straw bedding and a 6.25-m^2 outdoor area on a concrete floor. Pens were equipped with a heated nest only accessible to piglets. Fresh straw was added daily. Male piglets were not castrated, teeth were not clipped and tails were kept intact. If needed, cross-fostering was performed in the first three days of age (five of 12 litters were subjected to cross-fostering, with two adopted piglets on average [range: 1-3]). Piglets received daily access to solid feed from 20 days of age onwards. Piglets did not receive an iron injection because they were used in a project that explored whether daily access to soil or peat during the suckling period could be an applicable alternative strategy to the early iron injection to prevent risks of anaemia in suckling piglets raised in organic farms. Therefore, at about four days of age, litters were randomly allocated to one of two treatments (*i.e.* soil or peat), with six litters per treatment in total (*i.e.* three litters per treatment per cohort). Piglets received daily access to a small amount of sterilized soil or peat from 4 to 48 days of age. Soil and peat were distributed daily at 09:00 h in a small circular feeder located in the nest and not accessible to the sow. Daily rations of soil and peat per pen were 150 g from 4 to 12 days of age, 200 g from 13 to 26 days of age, and 250 g from 27 to 47 days of age. Apart from the access to soil or peat, animals from both groups were kept in the same husbandry conditions. The treatment effect was included in the statistical model (see section 2.3.1.), and because limited effects of treatment were found on the behaviours included in this study, animals from both treatments were kept in the study.

At about 40 days of age, six focal piglets (three males and three females) per litter were selected to be representative of intra-litter diversity for birthweight (1.52 ± 0.04 kg, [0.82-2.32 kg]), resulting in 72 focal piglets in total. Adopted piglets and piglets with health problems (diseases or lameness) were not included in the selection.

At 48 days of age, piglets were weaned and mixed with piglets from other litters. Per cohort, the 36 focal piglets were housed in two post-weaning pens. In each pen, the six focal littermates of three litters (18 piglets) were mixed together, and the pen was completed with piglets from three non-experimental litters balanced for sex and weight at weaning. This resulted in four post-weaning groups in total for the two cohorts, with 33 ± 2.58 piglets from six litters per pen. Non-focal piglets from the experimental litters were housed in additional non-experimental post-weaning pens. All post-weaning pens consisted of a covered 39-m^2 area with a concrete floor with deep straw bedding, and a 30-m^2 outdoor area with a concrete floor. Fresh straw was added weekly. Pens were equipped with two feeders and two drinking nipples. From weaning onwards, piglets had *ad libitum* access to a solid pelleted diet adapted to the nutrient requirements for weaned piglets and distributed automatically.

2.2. Measurements

2.2.1. Physiological measures

At 41 (*i.e.* 1 week before weaning), 49 (*i.e.* 24h after weaning) and 68 (*i.e.* end of the experiment) days of age, the 72 piglets were weighed, and at 41 and 49 days of age, blood was collected. The sampling procedure started at the same time (~09:00 h) on both days, and blood from the piglets was collected in a random order. Pigs were restrained in a prone position on a custom-made bed adapted to the size of the animal. The blood sampling procedure (including catching of the animal) was timed, and took less than 2 min per pig, thus limiting any potential effect of stress on physiological and behavioural measures. Blood was collected into one 5-mL Vacutainer EDTA and one 5-mL heparinized tube by jugular venepuncture and was kept on ice until processing.

Within one hour after sampling, blood cell counts (lymphocytes and neutrophils, thousand/mm^3; haemoglobin concentrations, g/dL) were measured in whole blood EDTA samples with a haematology automated cell counter calibrated for pigs (MS9; Melet Schloesing Laboratories, Osny, France). Whole blood EDTA samples were then centrifuged at 200 × g at room temperature for 10 min to obtain platelet-rich plasma (PRP). Samples of 200 µL of extracted PRP were completed with 800 µL phosphate buffer saline and centrifuged at 4500 × g for 10 min at 4°C. The supernatants were retrieved, the pellets were resuspended in 200 µL of distilled water and PRP samples were stored at -80°C until 5-HT analyses. The 5-HT concentrations were determined using an ultra-performance liquid chromatography (UPLC) apparatus (Waters Acquity Ultra Performance LC, Waters, Milford, MA, USA) coupled to two detectors (Acquity Tunable UV detector and Mass SQD detector; Waters, Milford, MA, USA) after derivatization of samples using the AccQ Tag Ultra method (MassTrak AAA; Waters, Milford, MA, USA). Norvaline (Sigma-

Aldrich, Saint Quentin Fallavier, France) was used as an internal standard. Concentrations of 5-HT were expressed in μmol/L PRP, and inter-assay coefficient of variation (CV) was 11%.

Heparinized blood samples were centrifuged at 1800 × g for 10 min at 4°C. Plasma was collected and stored at -20°C until analyses of inflammatory (haptoglobin) and oxidative stress markers (hydroperoxides). Haptoglobin (Tridelta Development Ltd, Maynooth, Ireland), and hydroperoxides generated by the peroxidation of lipids, proteins or nucleic acids (diacron Reactive Oxygen Metabolites , dROM kit, H&D srl, Parma, Italy) were assayed using commercial kits. All measurements were performed in duplicates using a multianalyzer apparatus (Konelab 20i, ThermoFisher Scientific, Courtaboeuf, France). The minimum concentration detectable for haptoglobin was 0.033 mg/mL, and the intra- and inter-assay CVs were 7 and 24 %, respectively. Concentrations of dROM were expressed in CARRU (Carratelli Unit, 1 CARRU = 0.08 mg H_2O_2/100 mL of sample) and intra- and inter-assay CVs were 6 and 8 %, respectively.

2.2.2. Behavioural measures

Immediately after blood sampling, the focal piglets were individually marked with a symbol sprayed on their back, and then video footage was continuously recorded for the analysis of behaviours at 42, 49 and 50 days of age. For the pre-weaning period, at 42 days of age, four 2-h sessions of observation (8:00-10:00 h, 11:00-13:00 h, 14:00-16:00 h, and 17:00-19:00 h) were analysed. For the post-weaning period, on day 49 and 50, two 3-h sessions of observation (12:30-15:30 h and 15:30-18:30 h) on both days were analysed.

Social interactions were scored during the pre- (day 42) and post-weaning (days 49 and 50) periods using the continuous all-occurrence behavioural sampling method and were expressed as frequencies (occurrences per hour). A total of four categories of social interactions were defined (**Table 1**): social exploration (nosing head, nosing body, snout-to-snout contact and gentle nudging), social aggression (head and shoulder knocks, aggressive bites and bite attempts), social locomotor play (chasing, climbing or pushing) and play-fight (mutual ramming and pushing). Behavioural activities were scored during the post-weaning period only (d49 and 50) using a 6-min scan sampling, resulting in 120 samples per pig in total, and were expressed as proportions of total scans. A total of eight categories of activities were identified (**Table 2**): being inactive, exploring the pen, walking, exploring pen mates (social nosing, nibbling and gentle nudging), interacting negatively with pen mates (aggression, mounting, belly nosing), playing (social and individual), ingesting (feed and water), and maintenance behaviours (scratching, urinating, defecating).

Table 1

Ethogram of social behaviours of piglets observed continuously before and after weaning and social mixing

Behaviour	Description
Social exploration	The pig touches the snout of the pen mate with its snout (nosing nose). The pig touches, gently rubs or licks the body (including its head, ears or tail) of a pen mate with its snout (nosing body). Includes licking and nibbling hairs or eyelashes. The pig does gentle pushes or up and down movements with its snout on the body of a pen mate (gently nudging). Usually occurs in bouts of behaviours in quick succession.
Social aggression	The pig gives a head or shoulder knock, i.e. strikes another pig with significant force, and/or aggressively bite any part of the body of a pen mate. Can occur in bouts of behaviours in quick succession. Can result in, but does not include active reciprocal fight.
Social locomotor play	The pig runs and chases other pigs intensely with rapid changes in direction (chasing). The pig drives its head or shoulders with minimal, moderate or substantial force at a target piglet, excluding frontal play invite (play invite, pushing). May cause the target to lose balance and fall over. The pig climbs or attempts to do so from the side or front of another pig (climbing). Play may be associated with barkings and gentle nudging of pen mates. Play is only scored once per playing bout. A playing bout is finished when the focal pig stops running, chasing or pushing other pigs for at least 10 sec or engages in another activity. Play is not associated with delivery or receipt of aggression and does not include pushing past other pigs restricting passage during locomotion, suckling at the udder or joining a resting pile of pigs.
Play-fight	The pig gives frontal head or shoulder knock with minimal, moderate or substantial force to another animal to invite it to play fight. Mutual ramming or pushing, with or without non-aggressive biting attempts.

Table 2

Ethogram of behavioural activities of piglets observed by scan sampling after weaning and social mixing

Behaviour	Description
Being inactive	The pig is standing, sitting, kneeling or lying without performing any other described behaviour.
Walking	The pig is walking without performing any other described behaviour.
Exploring pen	The pig is sniffing, touching with its snout, chewing or rooting (substrate on) the floor or any part of the pen. The pig is scraping its leg on the floor.
Ingesting	The pig has its snout in the feeder or in the drinking bowl. The pig is chewing while standing close to the feeder.
Exploring pen mates	The pig touches the snout of the pen mate with its snout (nosing nose). The pig touches, gently rubs, nibbles or licks the body or hairs of a pen mate with its snout (nosing body). The pig does gentle pushes or up and down movements with its snout on the body of a pen mate (gentle nudging). Usually occurs in bouts of behaviours in quick succession.
Interacting negatively with pen mates	The pig gives a head or shoulder knock, and/or aggressively bite any part of the body of a pen mate, or two pigs are engaged in a mutual fight (aggression). The pig is standing on its hind legs while having front legs on the body of a pen mate (mounting).
Belly nosing	The pig is rubbing the belly of a pen mate vigorously with up and down movements of the snout.
Playing	The pig is pivoting, rolling, sliding, or running around the pen alone (locomotor play) or with pen mates (social play). The pig is shaking its head while having straw in its mouth (object play).
Maintenance behaviours	The pig is rubbing its body against objects or pen mates. The pig is scratching its body with hind legs. The pig is defecating or urinating.

2.3. Statistical analyses

Data analysis was conducted using the statistical R version 4.0.0 (Team, 2020) with the packages 'lme4' version 1.1-26 (Bates et al., 2015), 'FactoMineR' version 2.3 (Husson et al., 2020), 'factorextra' version 1.0.7 (Kassambara and Mundt, 2020), and 'ggplot2' version 3.3.1 (Wickham et al., 2020).

2.3.1. Descriptive analyses of variables before and/or after weaning and social mixing

Of the 72 focal pigs selected from the 12 litters, two piglets (one male and one female piglet from two different litters) were excluded from the pre-weaning observations and four piglets (three female and one male piglet from four different litters) were excluded from the post-weaning observations because they could not be identified on the video recordings (*i.e.* erased markings), resulting in a final sample of 70 piglets before weaning and 68 piglets after weaning. Social interactions before and after weaning were expressed as frequencies (occurrences per hour). Behavioural activities after weaning were averaged over the two observation days (d49 and d50) and were expressed as proportions of total visible scans.

The effects of sex and period on social interactions (frequency of social exploration, aggression and total social interactions) and health-related blood variables were analysed using linear mixed-effect models with repeated measures including the fixed effects of sex, period (before *vs* after weaning), sex × period interaction, and pre-weaning treatment (soil *vs* peat), the random effects of litter nested within cohort, and the repeated effect of pig. Because play-fight and social locomotor play were not observed after weaning, these pre-weaning variables were analysed using linear mixed-effect models including the fixed effects of sex and treatment, and the random effects of litter nested within cohort. Because treatment effects were not the focus of this paper and only few significant effects of treatment were found (blood haemoglobin concentrations, soil: 9.43 ± 0.21 g/dL, peat: 11.2 ± 0.11 g/dL, $p < 0.001$; blood haptoglobin concentrations, soil: 1.24 ± 0.16 mg/mL, peat: 0.74 ± 0.0.11 mg/mL, $p = 0.03$), treatment effects are not discussed. Model residuals were visually inspected for normality and homoscedasticity, and if the residuals did not meet the assumptions for normal distribution and equality of variances, response variables were transformed to fit normal distribution (square root, arcsine square root, and log transformations were applied to skewed distributions of frequencies, proportions and concentrations, respectively). Data are presented as means ± SEM unless stated otherwise.

2.3.2. Identification of profiles of adaptation to weaning and social mixing.

A principal component analysis (PCA) was performed to analyse the correlational structure between post-weaning traits related to adaptation to weaning and social mixing. The 68 focal piglets observed after weaning were included as subjects, and 15 post-weaning variables were included as active variables: eight behavioural variables (frequency of social exploration and aggression, percentage of time spent being inactive, exploring the pen, walking, exploring pen mates, exhibiting negative social behaviours, and ingesting), six health-related blood variables (blood haptoglobin, hydroperoxide, and 5-HT concentrations, counts of lymphocytes and neutrophils, and lymphocyte-to-neutrophil ratio), and relative average daily gain (rADG, ADG corrected by the initial body weight at weaning; Le Floc'h et al., 2021) from weaning until 68 days of age. Sex, frequency of total social interactions and blood haemoglobin concentrations after weaning were included as supplemental variables. All active variables were subjected to a linear model with treatment and cohort as fixed effects to obtain their residuals, which were used for the PCA. The criteria for extracting principal components (PC) were an eigenvalue ≥ 1.5, cumulative percentage of variance ≥ 50% and visual inspection of the scree plot. The extracted PC were described with the variable residuals with loadings > 0.50 or < –0.50. A hierarchical clustering on principal components (HCPC) was then performed on the PC extracted from the PCA to identify clustered groups of pigs differing in their adaptive responses to weaning and social mixing. The Euclidean distance was used between individuals and the Ward's criterion was applied as clustering method (Lê et al., 2008).

2.3.3. Associations between early social-related variables and adaptation to weaning

Associations between each pre-weaning social-related variable (frequency of social exploration, aggression, social locomotor play, play-fight, and blood platelet 5-HT concentrations) and allocation to the clusters of adaptation to weaning were analysed with linear mixed-effect models including the fixed effect of cluster and the random effects of litter nested within cohort. As a complementary approach, effects of pre-weaning social-related variables on each behavioural and physiological response to weaning were analysed with linear mixed models including the fixed effects of pre-weaning social-related variables (frequency of social exploration, aggression, social locomotor play, play-fight, and platelet 5-HT concentrations) and sex, and the random effects of litter and post-weaning pen nested within cohort. A positive regression coefficient (β) indicated that the response and explanatory variables varied in the same direction and negative value indicated that they varied in the opposite direction. Frequency of total social interactions was included as an explanatory variable in the initial models, but was removed from the final models due to a high collinearity between this variable and other explanatory variables (social exploration, $r = 0.71$; social locomotor play, $r = 0.65$; and play-fight, $r = 0.79$). Moderate to very low correlations were found between other continuous explanatory variables ($r < 0.60$), which were thus all kept in the final models. Because strong correlations were found between hydroperoxide concentrations before and after weaning ($r = 0.81$), and between haemoglobin concentrations before and after weaning ($r = 0.80$), the pre-weaning values of these variables were added as covariates in their respective models. For all models, model residuals were visually inspected for normality and homoscedasticity, and if the residuals did not meet the assumptions for normal distribution and equality of variances, response variables were transformed to fit normal distribution. Data are presented as means ± SEM unless stated otherwise.

3. Results

3.1. Descriptive analysis of data

One week before weaning (d42), 4061 social interactions were scored continuously, that is on average 58.0 ± 3.68 [min: 4-max: 149] social interactions per pig ($n = 70$) and 7.25 ± 0.46 [0.50-18.6] social interactions per pig per hour. With a total of 2250 occurrences, social exploration of pen mates (4.02 ± 0.24 occurrences/hour) represented 55% of all social interactions, followed by play-fight interactions (2.16 ± 0.27 occurrences/hour, 30% of total), aggression (0.58 ± 0.06, 8%), and social locomotor play (0.49 ± 0.06, 7%). During the two days following weaning (d49 and d50), 4452 social interactions were scored, that is on average 65.5 ± 3.33 [7-160] social interactions per pig and 5.46 ± 0.28 [0.58-13.3] social interactions per pig per hour. With a total of 3498 occurrences, social exploration of pen mates represented 79% of all interactions (4.29 ± 0.22 occurrences/hour), and aggression represented 21% of all interactions (1.17 ± 0.15 occurrences/hour), while play-fight and social locomotor play were not observed during the two first days following weaning. Regardless of sex, aggression showed a 102% increase in the two days following weaning compared to one week before weaning ($p = 0.04$). Regardless of the period, males were involved in more social interactions overall (males: 7.13 ± 0.43, females: 5.44 ± 0.33, $p = 0.001$), and in more aggressions ($p < 0.001$). Males were also involved in more play-fight episodes than females before weaning ($p < 0.001$; **Fig. 1**).

Blood variables measured one week before and 24 hours after weaning are presented in **Table 3**. Minor effects of sex, period or both were found. Blood hydroperoxide concentrations increased significantly after weaning ($p = 0.002$), while blood haemoglobin concentrations were lower in females than in males before weaning ($p = 0.002$).

Analysis of behavioural activities by scan sampling in the two days following weaning showed that piglets spent on average 66% [min: 44-max: 89%] of total time (*i.e.* total visible scans) inactive, and little time engaged in other behavioural activities. They spent 15% [2.5-28%] of total time exploring the pen, 11% [2.5-28%] walking, 6.0% [0.8-18%] ingesting feed or water, 1.1% [0.0-3.5%] exploring pen mates, 0.6% [0.0-7.0%] negatively interacting with pen mates, and 0.1% [0.0-1.0%] in maintenance behaviours. Neither play nor belly nosing behaviours were observed by scan sampling during the two days following weaning.

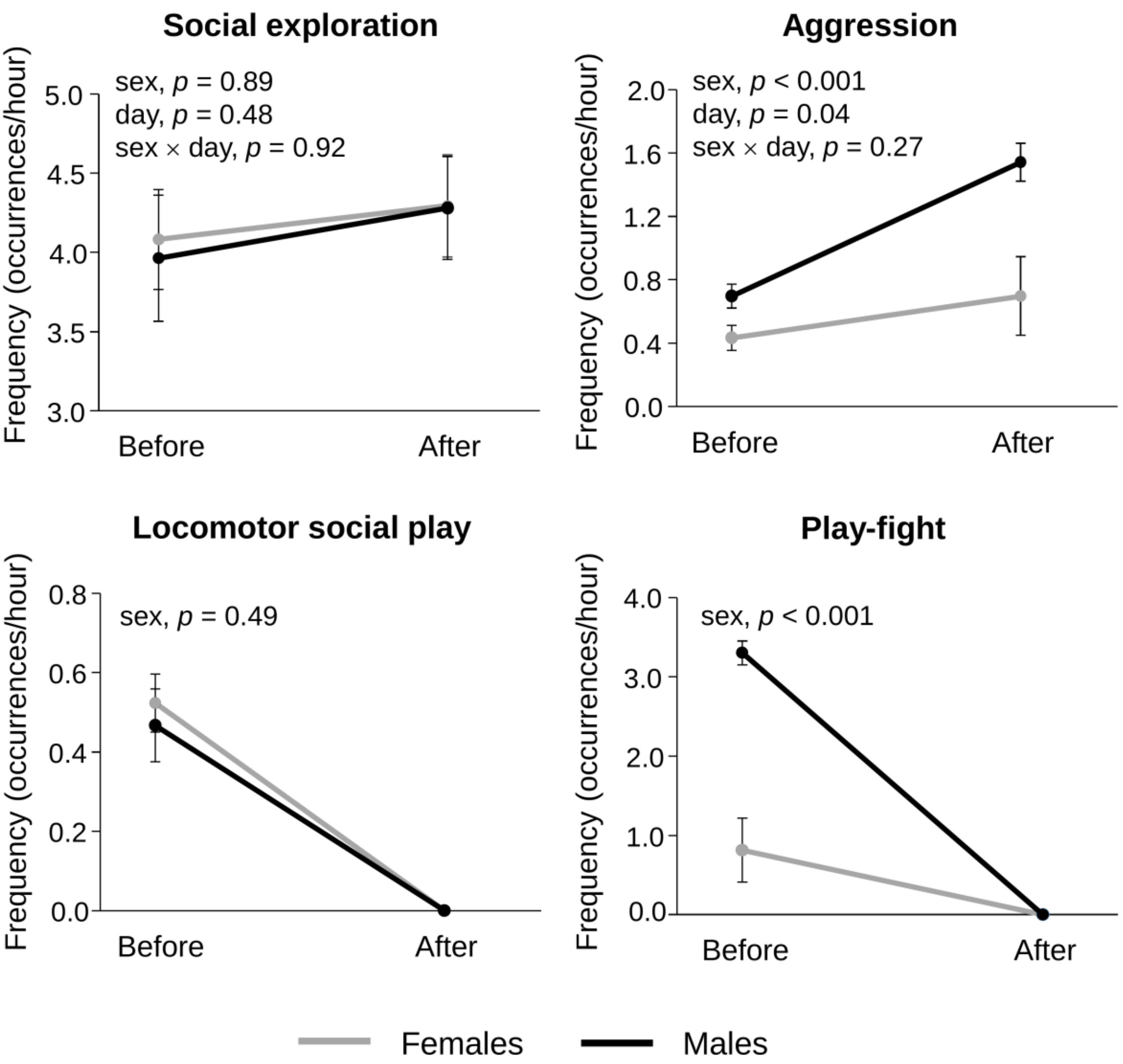

Fig. 1. Social interactions (occurrences per hour, means ± SEM) of male and female piglets observed continuously for 8 hours per day one week before weaning (before) and for 6 hours per day during the two days following weaning (after).

Table 3

Effects of sex and day on health-related blood parameters and performance of weaned piglets

	Pre-weaning		Post-weaning		*p*-value		
	Male	Female	Male	Female	day	sex	day × sex
Weight (kg)	10.6 ± 0.48	10.3 ± 0.51	12.9 ± 0.59	12.5 ± 0.58	0.005	ns	ns
Haptoglobin (mg/mL)	1.12 ± 0.24	1.37 ± 0.23	0.82 ± 0.15	0.65 ± 0.10	ns	ns	ns
Hydroperoxides (CARRU[1])	878 ± 28.6	874 ± 37.7	949 ± 29.6	897 ± 36.6	0.002	ns	ns
Lymphocytes (thousand/mm^3)	10.4 ± 0.59	9.41 ± 0.56	9.83 ± 0.54	9.33 ± 0.45	ns	ns	ns
Neutrophils (thousand/mm^3)	7.26 ± 0.47	6.16 ± 0.45	6.44 ± 0.36	6.39 ± 0.53	ns	ns	ns
Lymphocyte-to-neutrophil ratio	1.69 ± 0.15	1.80 ± 0.22	1.65 ± 0.11	1.70 ± 0.14	ns	ns	ns
Haemoglobin (g/dL)	10.6 ± 0.28[b]	9.71 ± 0.33[a]	10.4 ± 0.20[b]	10.3 ± 0.26[b]	ns	ns	0.004
5-HT (µmol/L PRP)	12.9 ± 1.49	11.5 ± 1.38	15.9 ± 1.46	15.5 ± 2.05	ns	ns	ns

[1] Carratelli Unit, 1 CARRU = 0.08 H_2O_2/100 mL of sample

[2] Different letters indicate a significant difference between groups ($p < 0.05$)

3.2. *Identification of profiles of adaptation to weaning and social mixing*

A total of three principal components (PC) explaining 50% of the total variance were extracted from the PCA performed on the 68 piglets observed after weaning (**Table S1**). The first PC accounted for 21% of the total variance. The frequency of social exploration (loading: 0.64), the percentage of time spent exploring the pen (0.60) and walking (0.50) and the lymphocyte-to-neutrophil ratio (0.55) loaded positively, while the percentage of time spent inactive (loading: -0.73) and blood concentrations in haptoglobin (-0.66) loaded negatively on the component, which was thus labelled *'(social) exploration* vs *inactivity & inflammation'*. The second PC accounted for 16 % of the total variance. The count of neutrophils (0.69) loaded positively, while the percentage of time spent inactive (-0.59) and the lymphocyte-to-neutrophil ratio (-0.58) loaded negatively on the second component, which was thus labelled '*neutrophil count* vs *inactivity'*. The third PC accounted for 13% of the total variance. Frequency of aggression (continuous observations, 0.76) and time spent interacting negatively with pen mates (scan sampling observations, 0.78) loaded positively on the third component, which was thus labelled '*negative social behaviours'*.

The hierarchical clustering analysis was performed on the three extracted PC to identify clustered groups of pigs based on responses to weaning. We extracted three clusters from the HCPC (**Fig. 2**). Of the 68 piglets included in the PCA, 11 were in cluster 1 (16% of all piglets), 31 in cluster 2 (46% of all piglets) and 26 in cluster 3 (38% of all piglets). Compared to all animals, animals from cluster 1 had lower coordinates on PC 1 ('*(social) exploration vs inactivity & inflammation'*, $p < 0.001$) and PC 3 ('*negative social behaviours*', $p < 0.01$). Animals in cluster 1 showed lower frequencies of social exploration and total social interactions, spent less time exploring pen mates or the pen and spent more time being inactive. They had higher concentrations of blood haptoglobin and hydroperoxides, greater counts of neutrophils, a lower lymphocyte-to-neutrophil ratio and a lower rADG ($p < 0.01$ for all). Cluster 1 thus represented '*unhealthy inactive animals'*. Compared to all piglets, animals from cluster 2 had higher coordinates on PC 3 ('*negative social behaviours*', $p < 0.001$) and lower coordinates on PC 2 ('*neutrophil count* vs *inactivity',* $p < 0.001$). Animals in cluster 2 spent more time inactive and interacting negatively with pen mates, and spent less time walking and exploring the pen. They had lower concentrations of blood haptoglobin, 5-HT, and hydroperoxides, fewer counts of neutrophils, a higher lymphocyte-to-neutrophil ratio, and greater rADG. Cluster 2 thus represented '*healthy inactive aggressors'*. Compared to all animals, animals from cluster 3 had higher coordinates on PC 1 ('*(social) exploration vs inactivity & inflammation*', $p < 0.001$) and PC 2 ('*neutrophil count* vs *inactivity',* $p < 0.001$), and lower coordinates on PC 3 ('*negative social behaviours',* $p < 0.05$). Animals in cluster 3 were characterised by higher frequencies of social exploration, spent more time exploring the pen and pen mates and walking, but less time interacting negatively with pen mates and being inactive. They also had higher blood 5-HT concentrations. Cluster 3 thus represented '*healthy active and affiliative animals*'. Clusters were not characterised by sex differences.

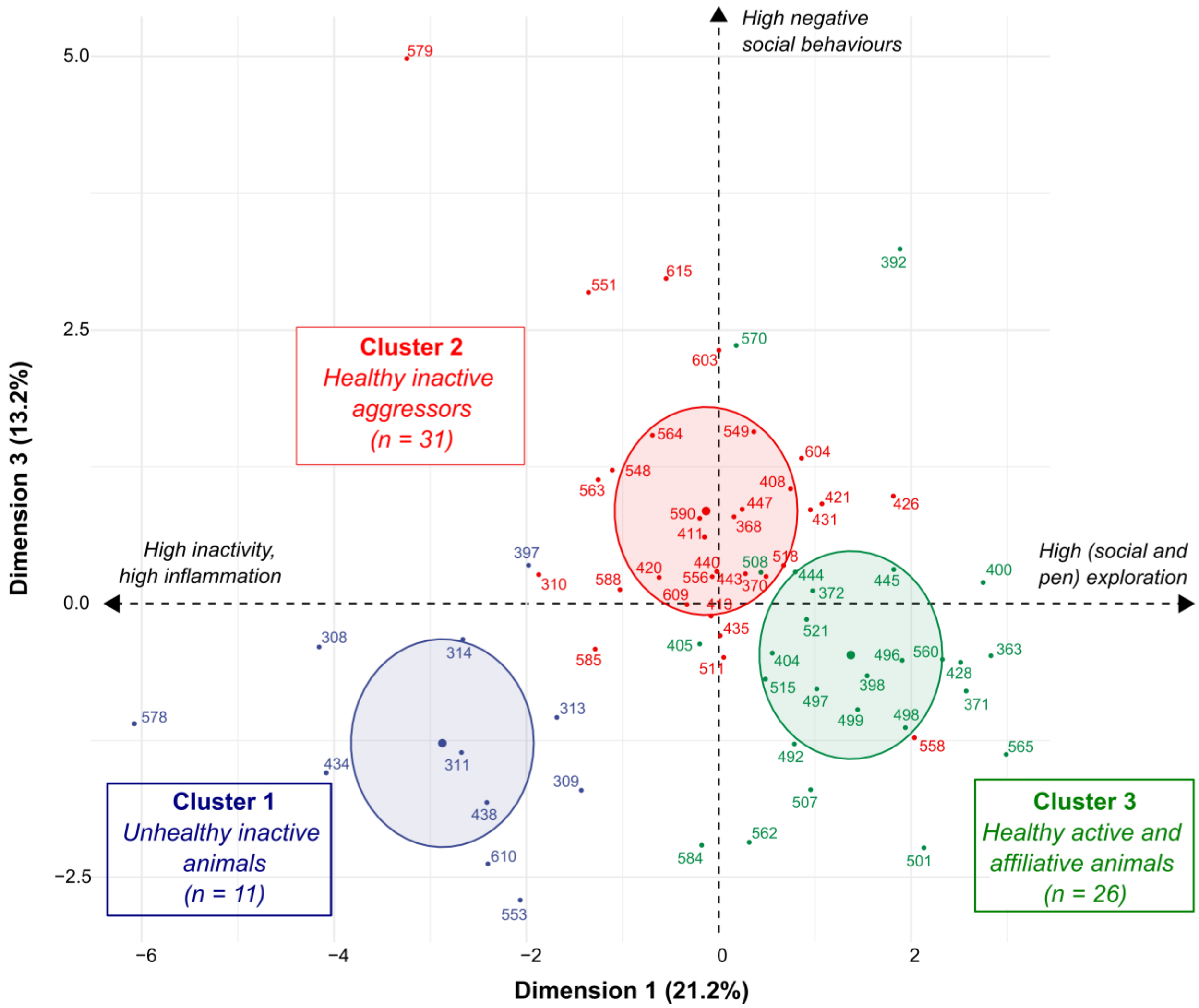

Fig. 2. Three clustered groups of piglets differing in their behavioural and physiological responses to weaning and social mixing, according to (social) exploration, inactivity and inflammation (dimension 1) and negative social behaviours (dimension 3). Dimension 2 (behavioural activity and neutrophil count) is not shown. The three dimensions were extracted from a Principal Component Analysis (PCA) computed on the social, behavioural and physiological responses of 68 piglets measured after weaning. Hierarchical Clustering on Principal Components was then performed on the dimensions extracted from the PCA to identify clustered groups of pigs differing in their adaptive responses to weaning and social mixing. Ellipses of the clusters are plotted according to the Euclidian distance.

3.3. Association between pre-weaning social-related variables and adaptation to weaning and social mixing

3.3.1. Association of pre-weaning social-related variables with the profiles of adaptation to weaning

Animals with different profiles of adaptation to social mixing (*i.e.* from different clusters) differed in the social behaviours they exhibited one week before weaning, and, notably, in the frequency of social exploration (p =0.005), aggression (p = 0.04), social locomotor play (p = 0.03) and, to a lesser extent, play-fight (p = 0.06, **Fig. 3**). Post-hoc analyses showed that *unhealthy inactive animals* at weaning (cluster 1) aggressed their pen mates less (p = 0.03), and tended to explore them less (p = 0.06) and to be involved in fewer play-fight episodes (p = 0.06) one week before weaning than *healthy inactive aggressors* (cluster 2). *Unhealthy inactive animals* at weaning (cluster 1) also explored their pen mates less (p = 0.007) and were involved in fewer social locomotor play episodes (p = 0.02) one week before weaning than *healthy active affiliative animals* (cluster 3). Animals from clusters 2 and 3, however, did not differ in their pre-weaning social behaviours.

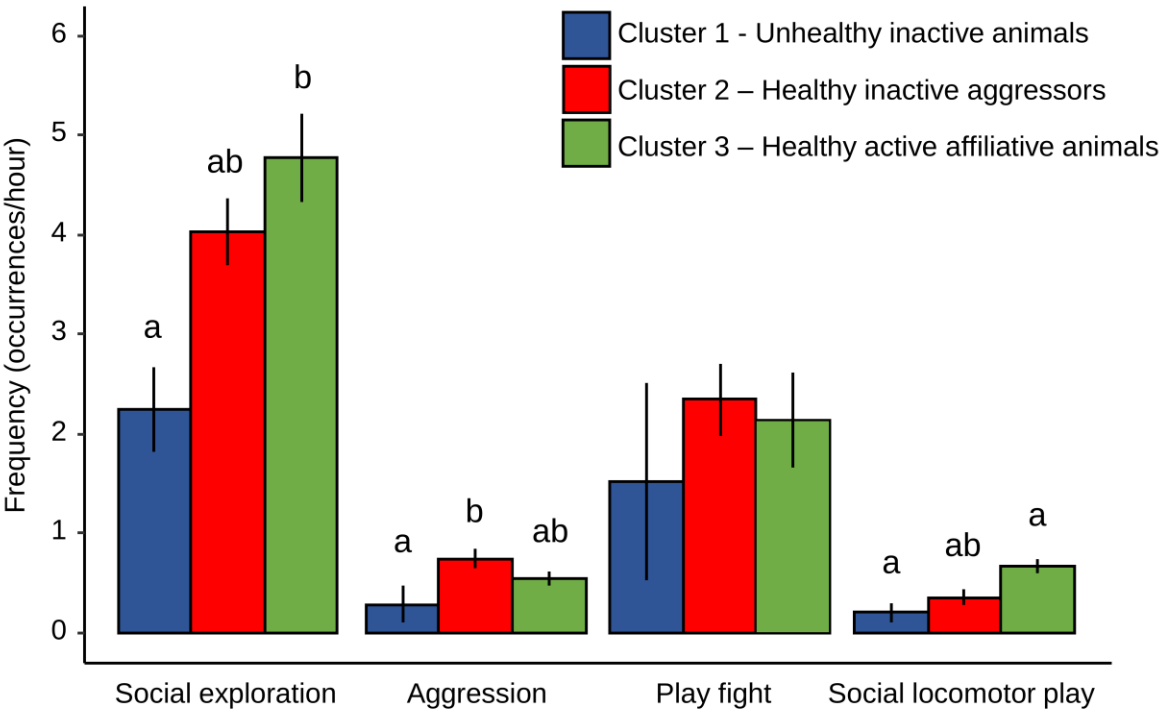

Fig. 3. Associations between the frequency of social behaviours measured one week before weaning and the allocation to clusters reflecting divergent profiles of adaption to weaning and social mixing in piglets (cluster 1, n = 11 piglets; cluster 2, n = 31 piglets, cluster 3, n = 26 piglets). Two different letters indicate significant differences between clusters for a single behaviour ($p < 0.05$).

3.3.2. Association of pre-weaning social-related variables with variables measured after weaning

Detailed results from the covariance analyses are presented in **Table 4,** and only statistically significant results are listed below. When taken separately, some behavioural and/or physiological responses to weaning were associated with social-related variables measured one week before weaning.

Exploration of littermates one week before weaning was negatively associated with the time spent inactive during the two days following weaning (β = -0.01, p = 0.03), and tended to be positively associated with total social interactions (β = 0.06, p = 0.06) and the time spent exploring pen mates (β = 0.19, p = 0.08) after weaning. Exploration of littermates before weaning was also positively associated with blood haemoglobin concentration measured 24h after weaning (β = 0.12, p = 0.01). Aggression of littermates one week before weaning was negatively associated with the time spent walking during the two days following weaning (β = 0.19, p = 0.08), and tended to be positively associated with the time spent inactive after weaning (β = 0.04, p = 0.08). Aggression of littermates before weaning was also positively associated with blood lymphocyte count measured 24h after weaning (β = 1.72, p = 0.04), and tended to be negatively associated with blood haemoglobin concentration after weaning (β = -0.37, p = 0.08). Social locomotor play one week before weaning was positively associated with the time spent walking during the two days following weaning (β = 0.09, p = 0.009) and with hydroperoxide concentration measured 24h after weaning (β = 0.10, p = 0.05). Finally, blood 5-HT concentration one week before weaning was negatively associated with aggression of pen mates during the two days following weaning (β = -0.02, p = 0.01), and positively associated with blood 5-HT concentration measure 24h after weaning (β = 0.44, p = 0.009).

Table 4

Effects (illustrated by coefficients of regression) of social-related parameters measured one week before weaning (d42) on the social behaviours, behavioural activities, blood parameters and performance of piglets measured after weaning and social mixing

(Post-weaning) response variables	Explanatory (pre-weaning) variables				
	Social exploration (occurrences/hour)	Aggression (occurrences /hour)	Play-fight (occurrences /hour)	Social locomotor play (occurrences /hour)	5-HT (µmol/L PRP[a])
Social interactions (occurrences/hour)					
Total interactions	***0.06#***[b]	0.08	-0.01	-0.23	-0.01
Social exploration	***0.19#***	0.14	-0.07	-0.72	-0.009
Aggression	0.02	0.13	-0.007	-0.08	**-0.02****
Behavioural activity (proportion of total visible scans)					
Being inactive	**-0.01***	***0.04#***	0.008	-0.01	0.002
Exploring the pen	0.005	-0.01	0.002	0.003	<0.001
Walking	0.003	**-0.04***	-0.01	**0.09****	<-0.001
Exploring pen mates	<0.001	0.002	0.001	-0.004	<-0.001
Interacting negatively	0.002	0.01	-0.002	-0.01	-0.002
Ingesting	0.007	-0.02	-0.006	0.004	<-0.001
Blood parameters					
Hydroperoxides[c] (CARRU[d])	-0.006	0.04	-0.005	**0.10***	-0.003
Haptoglobin (mg/mL)	-0.04	0.01	-0.01	-0.15	0.002
Lymphocytes (thousand/mm^3)	-0.01	**1.72***	0.15	-0.007	-0.007
Neutrophils (thousand/mm^3)	-0.04	0.04	0.02	0.09	-0.009
Lymphocyte-to-neutrophil ratio	0.03	0.17	-0.003	0.01	0.01
Haemoglobin[e] (g/dL)	**0.12****	***-0.37#***	0.02	-0.32	-0.02
5-HT (µmol/L PRP[e])	0.68	-2.17	0.005	-2.58	**0.44****
Performance					
rADG[f] (g/d)	0.45	3.03	0.71	4.18	0.19

[a] PRP = platelet-rich plasma

[b] Coeffient of regression (β) calculated from 68 pigs. A positive coefficient indicates that the response and explanatory variables vary in the same direction and negative coefficient indicates that they vary in the opposite direction. # $0.10 > p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

[c] This model included blood hydroxperoxide concentrations one week before weaning as covariate ($\beta = 0.0007$, $p < 0.001$)

[d] Carratelli Unit, 1 CARRU = 0.08 H_2O_2/100 mL of sample

[e] This model included blood haemoglobin concentrations one week before weaning as covariate ($\beta = 0.60$, $p < 0.001$)

[f] rADG = relative average daily gain, *i.e.* weight gain from weaning to 20 days after weaning divided by weight at weaning

4. Discussion

This study aimed to characterise distinctive profiles of adaptation to weaning and social mixing in piglets based on behavioural responses to weaning and physiological indicators of health, and to determine whether social variables prior to weaning were associated with these adaptive responses to weaning. In our study, weaning had a minor impact on physiological variables, with only a rise in blood hydroperoxide concentrations, but affected social behaviours, as shown by a rise in aggression and an inhibition in play behaviours in the two days following weaning. These results agree with prior research showing that social mixing is usually associated with intense fights to establish a new hierarchy (Meese and Ewbank 1973), and with a rise in markers of oxidative stress (Sauerwein et al., 2007; Buchet et al., 2017). Contrarily to others, we failed to report a rise in blood haptoglobin concentrations, but this rise was typically measured five days or more after weaning (Sauerwein et al., 2007; Montagne et al., 2022). Furthermore, piglets spent most of their time inactive, and little time engaged in other behavioural activities, such as pen exploration, locomotion or social interactions, while play was not observed during the two days following weaning. Accordingly, early-weaned piglets have been found to explore their environment less (Worobec et al., 1999), show a major drop in play behaviour (Donaldson et al., 2002) and spend more time lying or resting directly after weaning (Metz and Gonyou, 1990). We, however, did not observe belly nosing, as reported in early-weaned piglets raised in a poor environment (Widowski et al., 2008). The onset of belly nosing typically appears around four days after weaning (Metz and Gonyou, 1990; Worobec et al., 1999) and is usually associated with early weaning (less than 4 weeks of age; Worobec et al., 1999). The absence of belly nosing and the lack of variation in most of the blood variables are likely explained by the “close to optimal” environmental conditions of our study (weaning at 7 weeks of age, moderate social challenge, low density, access to a foraging substrate and outdoor area; Oostindjer et al., 2011). Therefore, although weaning remains a source of stress for piglets, optimal environmental and social conditions may partially alleviate the negative impact of weaning and social mixing on the behaviour and health of the piglets.

Males emitted more aggression and play-fight than females, but comparable levels of other social behaviours, supporting the existence of sexual dimorphism in agonistic behaviours in pigs (Rydhmer et al., 2006; Melotti et al., 2011). Social exploration remained more prevalent than agonistic behaviours both before (55% of all interactions) and after social mixing (78% of all interactions), as reported by others (Beattie et al., 2000; Camerlink et al., 2021; Clouard et al., 2022). Further research should consider investigating whether social nosing after social mixing occurs mainly between piglets originating from the same litter or from different litters to elucidate the function of social nosing in unstable social groups. In pigs, social nosing is assumed to contribute to affiliation and social cohesion, and to generate positive emotional states (Uvnas-Moberg, 1998; Camerlink and Turner, 2013; Camerlink et al., 2014; Camerlink et al., 2016). Therefore, one hypothesis would be that piglets primarily nosed littermates to alleviate the stress caused by mixing and to maintain social cohesion between already-known partners. Although social nosing has been found to be largely unrelated to received aggression (Camerlink and Turner, 2013), an alternate hypothesis would be that social nosing occurred between piglets from different litters to help in social recognition, favour rapid group cohesion and thus minimise aggressions.

Despite minor effects of weaning and social mixing, the multivariate analysis revealed three clusters of pigs differing in a variety of behavioural and physiological responses to weaning. Piglets from cluster 1 were characterised by variables indicative of degraded health status, and were thus labelled as *unhealthy inactive piglets*. They notably had a lower lymphocyte to neutrophil ratio, a variable which transiently drops at weaning or other stressful situations (Puppe et al., 1997; Sutherland et al., 2009), and is closely associated with the endocrine stress response (Dhabhar et al., 1995). They also had a lower growth, and higher blood levels of markers of oxidative stress and inflammation, which are usually associated with poor weaning conditions (Buchet et al., 2017). Finally, they displayed lower levels of social and environmental exploration. In a previous study focusing on the suckling period, we identified a cluster of socially inactive piglets characterised by low levels of (social and pen) exploration and activity and higher concentration of blood haptoglobin (Clouard et al., 2022). Therefore, we hypothesize that this association reflects a mild form of sickness behaviour, due to subclinical health problems, in the animals with the highest haptoglobin concentrations (Hennessy et al., 2014). Notably, *unhealthy inactive* piglets already showed lower frequencies of social exploration, aggression and social locomotor play one week before weaning than *healthy inactive aggressors* or *healthy active affiliative animals*. This low social motivation may reflect sickness behaviour due to subclinical health problems that were present before weaning. Since haptoglobin can remain elevated in the plasma for several days (Heegaard et al., 1998; Pomorska-Mól et al., 2012), the greater haptoglobin concentrations measured in those animals one day after weaning may reflect a poorer health status of piglets before weaning, which may have been worsened by social and nutritional stress at weaning.

In addition to the cluster of *unhealthy inactive animals*, we identified two other clusters, which seem to illustrate contrasted social profiles in healthy animals. *Healthy inactive aggressors* (cluster 2) was characterised by low levels of blood markers of inflammation and oxidative stress, a lot of time spent interacting negatively with pen mates, and little time spent being active, exploring the pen or walking. *Healthy active affiliative animals* (cluster 3), on the other hand, were characterised by high levels of positive social exploration, activity, locomotion and pen exploration, but low levels of negative social interactions. The identification of clusters of animals differing in their level of positive social exploration and aggression suggests that social exploration and aggression reflect distinct dimensions of sociality in pigs (Forkman et al., 1995). Consistent with this postulate, 'social exploration' and 'aggression' loaded on different axes of the PCA, which supports recent findings in suckling piglets (Clouard et al., 2022). Altogether, the identification of three clusters differing in health status and social behaviours after weaning suggests that responses of pigs to weaning and social mixing might result not only from their general fitness during the challenge, but also from their intrinsic social coping style. However, these coping styles could not be predicted by the pre-weaning social behaviours. We thus conclude that, while pre-weaning social behaviours may help in identifying animals with pre-weaning health issues which persisted after weaning, they may not be strong predictors of divergent adaptive capacity to weaning in healthy animals.

In addition to being associated with distinct profiles of adaption to weaning, pre-weaning social behaviours were also associated with independent behavioural and physiological responses to weaning. First, piglets that explored littermates more prior to weaning had the greater concentrations in blood haemoglobin and were more active after weaning, with a trend for higher frequencies of social interactions and social nosing after weaning. Because of the availability of substrate to forage, we posit that social exploration of pen mates did not only reflect a redirected motivation to explore (Weller et al., 2019), but also an intrinsic motivation for social contacts. Although these results need to be confirmed on a larger sample of animals, a high motivation to interact positively with pen mates before weaning may be an early indicator of good health and high levels of (affiliative social) activity following weaning (Worobec et al., 1999).

Second, piglets that were involved in higher number of locomotor play episodes one week before weaning spent more time walking after weaning, which may indicate that these piglets had a higher motivation for physical activity and a more active style. Alternatively, higher levels of walking after weaning may reflect the importance of early (locomotor) play in immature animals for the stimulation of muscle and bone development (Newberry et al., 1988; Horback, 2014). Surprisingly, piglets involved in a higher number of locomotor play episodes before weaning also had greater concentrations of hydroperoxides after weaning. Although the underlying cause of this association remains to be elucidated, piglets that played more just before weaning might have been more stressed by weaning and social mixing, which is reflected by the abrupt cessation of play after weaning. As high levels of glucocorticoids during (social) stress are typically associated with elevated oxidative stress (Costantini et al., 2011), these piglets might have been more susceptible to suffer from oxidative stress after weaning. However, because strong correlations were found between hydroperoxide concentrations before and after weaning, the greater hydroperoxide concentrations in these animals might be due to a different cause other than weaning. As physical exercise has been found to generate oxidative products, high hydroperoxide levels might be related to higher physical activity, as suggested by their higher levels of locomotor play before weaning and walking after weaning, (Urso and Clarkson, 2003).

Some anecdotal associations were also highlighted between pre-weaning aggression and responses to weaning. Piglets that were more aggressive before weaning notably had greater counts of lymphocytes and spent less time walking after weaning. After increased corticosteroid concentrations, a transient decrease in blood lymphocyte counts, paired with an increase in neutrophil counts, may be observed and is assumed to reflect the effect of stress on immune cell trafficking (Dhabhar et al., 1995). Our data may therefore reflect an attenuated stress response to weaning in piglets with the highest levels of pre-weaning aggression. However, although aggression or dominance status have been found to influence blood cell numbers and lymphocyte functions in rats (Stefanski, 2000) and pigs (Hjarvard et al., 2009), the association between lymphocyte counts and aggressiveness has never been reported in the literature and thus warrants further investigation.

In our study, pre- and post-weaning blood concentrations of 5-HT were positively correlated, and were consistent with concentrations reported in sows (Poletto et al., 2010) and weaned piglets (11 µmol/L; Rius et al., 2018). Furthermore, piglets with greater blood concentrations of 5-HT one week before weaning were less aggressive during the two days following weaning. This result agrees with prior reports showing a negative relationship between peripheral 5-HT concentration and agonistic behaviours in dogs (Rosado et al., 2010) and gilts (Poletto et al., 2010). Although further research is warranted to confirm these results, we argue that peripheral 5-HT concentrations may be a valid biomarker of aggression (*i.e.* actual engagement in aggression) or aggressiveness (*i.e.* the inclination to deliver aggression) in pigs, and may help to predict the emergence of episodes of exacerbated aggression after social mixing.

5. Conclusions

Our study revealed the existence of three distinct profiles of adaptation to weaning and social mixing in pigs (*unhealthy inactive animals*, *healthy inactive aggressors* and *healthy active affiliative animals*), characterised by contrasted health status and distinct behavioural responses to social mixing. These profiles of adaptive responses to weaning and social mixing seem to result not only from the piglets' general fitness before and during the challenge, but also from their intrinsic social characteristics. Furthermore, pre-weaning social variables, such as social nosing, social locomotor play, or blood 5-HT concentrations, may be relevant predictors of piglets' adaptive responses to a social challenge at weaning, and deserve more research attention. .

CREdiT authorship contribution statement

Caroline Clouard, Elodie Merlot, Armelle Prunier: Designed the study; **Caroline Clouard**: Collected the data; **Caroline Clouard, Héloïse Vesque-Annear**: Analysed the behavioural data; **Rémi Resmond, Caroline Clouard, Héloïse Vesque-Annear:** Conducted the statistical analyses; **Caroline Clouard:** Drafted and revised the manuscript; **Elodie Merlot, Armelle Prunier, Rémi Resmond:** Reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no know competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflict of interest

The authors declare no conflict of interest that would influence the analysis of the data nor presentation of the results.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.applanim.2023.105833.

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