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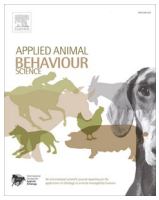

Exploration of skin redness and immunoglobulin A as markers of the affective states of hens

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ABSTRACT

Non-invasive markers of affective states can help understanding animals' perception of situations and improving their welfare. These markers are scarce in avian species. In this study, we investigate the potential relation between alterations in facial skin redness in hens and their corresponding affective states. Six hens were filmed in both naturally unfolding scenarios and controlled tests designed to elicit various affective states. The facial skin redness was measured from images extracted from the videos. Our observations revealed that hens exhibited the highest degree of facial skin redness in negative situations of high arousal, a high redness in positive situations of high arousal, and the lowest in positive situations of low arousal. In a second study, we further examined whether facial skin redness and secretory immunoglobulin A (S-IgA) can serve as markers for the quality of the human-animal relationship. Two groups of hens, one habituated to humans (n=13) and one non-habituated (n=12), were compared for general fearfulness in an open field test and for fear of humans in a reactivity to human test. In the open-field test, there were no statistical differences in general fearfulness, facial skin redness or S-IgA concentrations between both groups. However, habituated hens exhibited significantly lower fearfulness and facial skin redness in the presence of humans compared to non-habituated hens in the reactivity to human test. Additionally, habituated hens showed significant lower S-IgA concentration in lachrymal fluid in the presence of humans, with no significant differences in saliva or cloacal samples. We propose that changes in facial skin redness reflect variations in affective states and can be used as a marker for assessing the quality of the human-hen relationship. The relationship between S-IgA concentrations and affective states requires further investigation.

1. Introduction

Understanding how animals express affective states is a fundamental step toward comprehending their sentience. This comprehension, in turn, holds significant implications for advancing the science of animal welfare. Affective states, as defined by Mendl and Paul (2020), encompass both short-lived emotions and moods, the former being responses to specific stimuli and the latter representing longer-term free-floating states. To define affective states, a bi-dimensional model incorporating the valence axis (positive/pleasant to negative/unpleasant) and the arousal axis (intensity variation) has been widely adopted (Mendl et al., 2010; Russell, 2003). This model divides affective states into four categories: 1) positive affective states of high arousal associated with reward acquisition, 2) positive affective states of low arousal associated with calm and contentment, 3) negative affective states of high arousal associated with threat and 4) negative affective state of low arousal associated with sadness or depression. Despite the importance of affective states, there is a substantial deficit in non-invasive indicators, particularly for avian species.

The behavioral expression of emotions is widely studied through facial expressions (i.e. facial movements generated by the contraction of facial muscles) and movements such as ear posture or eye shape in

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various mammal species: primates, cats, horses, rodents, sheep, cattle, goats, pigs, and dogs (Descovich et al., 2017; Lansade et al., 2018; Neethirajan et al., 2021; Waller et al., 2020). Beyond facial movements, visible changes such as blushing in humans, indicating blood flow variations, also vary with affective states (Benitez-Quiroz et al., 2018; Thorstenson et al., 2019, 2018). While facial blushing was traditionally considered a distinctly human expression (Darwin, 1872), recent research has identified rapid changes in facial redness in certain birds with bare skin on their faces (Bamford et al., 2010; Bertin et al., 2023, 2018; Negro et al., 2006). For example, lappet-faced vultures (*Necrosyrtes monachus;* Bamford et al., 2010) and captive blue and yellow macaws (*Ara ararauna;* Bertin et al., 2023, 2018) were found to exhibit changes in facial redness in response to a negative or positive interaction, respectively. This suggests a potential link between facial redness and affective states in birds.

In this study, our primary objective was to investigate whether transient changes in the facial skin redness of domestic hens could serve as an indicator of their affective states.

Our secondary objective was to assess whether alterations in facial skin redness could be used to infer the quality of the human-animal relationship. In domestic animals, including poultry, the presence of humans often induces fear, impacting their health and productivity (Acharya et al., 2022; Jones, 1996; Waiblinger et al., 2006; Zulkifli, 2013). The behavior of caretakers significantly influences the human-animal relationship. For example, regular gentle handling reduces the fear of humans (Graml et al., 2008; Jones, 1996). However, there is only few markers for evaluating the human-bird relationship, such as the distance maintained between the birds and humans or with protocols like touch tests or the Qualitative Behaviour Assessment (Jones, 1996; Papageorgiou et al., 2023; Waiblinger et al., 2006), emphasizing the need for more indicators.

The third objective was to explore whether the quality of a human-animal relationship could also be measured with another non-invasive marker, the secretory immunoglobulin A (S-IgA). S-IgA, found at mucosal surfaces, plays a crucial role in protecting against diseases (Lamm, 1988; Staley et al., 2018). It can be collected non-invasively, most commonly from feces and saliva (Gourkow et al., 2014; Hucklebridge et al., 2000; Lv et al., 2018; McCraty et al., 1996). In the feces, S-IgA concentrations reveal an accumulation of IgA before defecation. In contrast, S-IgA measured in saliva is the result of only a short-term secretion (Staley et al., 2018). Studies have demonstrated that S-IgA concentrations vary based on affective states, offering insights into the potential relationship between emotional experiences and health (Gourkow et al., 2014; Hucklebridge et al., 2000; McCraty et al., 1996; Staley et al., 2018). In avian species, in addition to feces and saliva, S-IgA are also measurable in cloacal and lachrymal fluids (Merino-Guzmán et al., 2017), but the relation to affective states remains to be explored.

Overall, our study aimed to overcome the lack of non-invasive indicators of the affective state of hens and of the quality of their relationship with humans. To that end, in a first experiment, we investigated variations in facial redness under different valenced and aroused affective states. We expected to observe variations in facial redness depending on the emotional experiences. In a second experiment, we investigate the behavior, facial redness and S-IgA concentrations between hens habituated to human and non-habituated hens during an open field test, assessing general fearfulness, and a reactivity to human test. We expected a divergence specifically in the appraisal of human presence, and not on general fearfulness, translating into differences in fear related behaviors, facial redness and S-IgA concentrations between the two groups.

2. Materials and methods

2.1. Experiment 1: facial redness in contrasted emotional situations

2.1.1. Animals and housing

Six 3–4 months old hens (Sussex) identified with a leg colored ring were reared in a 363 m^2 wooded outdoor range covered with grass with free access to a hen house (width=154 cm, length=80 cm, height in front=80 cm, height in back=40 cm, with perches inside) (37800 Sainte Maure de Touraine, France). Water and feed were provided *ad libitum*.

2.1.2. Behavioral observations

The study involved the filming of hens in both spontaneous situations and in tests designed to evoke various emotional valence and arousal levels. The observation period lasted three consecutive weeks between 10 am and 5 pm (performed in summer days - September 2020 - with limited temperature variations as sun rose at approximately 7:30 am and set around 8 pm) and two Sony FDR-AX53 4 K cameras were used, with the outdoor automatic white balance function activated. The selection of spontaneous behaviors and tests, considered to be associated with specific affective state, was guided by the existing literature (Jones, 1996; McGrath et al., 2016; Mendl et al., 2010; Papageorgiou et al., 2023; Richardson et al., 2016), as well as personal observations:

1. Calm states (positive valence and low arousal level): Resting (hen lying with eyes opened or closed), Preening, and Feeding (hen consuming usual feed or grass).

2. Exciting and rewarding states (positive valence and high arousal level): Dustbathing and Rewarding Tests with mealworms.

3. Fear-related states (negative valence and high arousal): Capture Tests with manual restraint.

For each hen and spontaneous situation, approximately 20 minutes of film were recorded. The two behavioral tests, Capture Test then Rewarding Test, were conducted during the third week of observations.

The Capture Test involved individual hens being caught by the experimenter, who restrained the wings with two hands, and filming for 1 minute by a second experimenter. This test was repeated twice on the same day, with roles reversed between experimenters during the morning and afternoon sessions.

The Rewarding Test was conducted in a wire-mesh enclosure (length=118 cm, width=59 cm, height=58 cm) surrounded by cardboard to prevent visual contact with conspecifics (height=33 cm). The hens had free access to the enclosure 72 h before the test and were habituated to eat mealworms from a transparent glass dish (diameter: 70 mm, height: 40 mm) placed inside the enclosure. Each hen was tested individually with a transparent glass dish containing mealworms and wood shavings placed in the middle of the test arena. The filming started when the hen freely entered the enclosure and lasted for 2 minutes and 30 seconds. If a hen refused to enter but remained close to the enclosure, the test was performed at its entrance. The test was repeated twice on separate days. On the first day, four out of six hens were tested, the two other hens refused to approach the enclosure. On the second day, the six hens were tested, including two at the entrance of the enclosure.

2.1.3. Extraction of images and measure of facial redness

The process of extracting redness from still frames from hen profiles involved multiple steps to ensure robustness. The following procedures were followed, using python scripts and fiji macros (Schindelin et al., 2012):

a/ Evaluating the presence of hen profiles, in images extracted at interval of 2 seconds from films, by using a deep learning model detecting hen profile in images.

b/ Randomly sampling 20 images for each hen and situation by maximizing the number of films from which they come.

c/ Extracting the mean red (R), blue (B), and green (G) values for each bare skin region of the hen face (comb, cheek, ear lobe and wattle). For this, we used a fiji macro allowing the user to position 10×10 pixels

squares on the four regions of interest and on the white feathers for each image. As far as possible, three squares were positioned on each region and one for the white feathers (Fig. 1).

d/ Correcting the white balance of the images post acquisition by using the white of the feather. We follow a macro available on GitHub that balances RGB color based on a selected region (Patrice Mascalchi, 2017; https://github.com/pmascalchi/ImageJ_Auto-white-balance-correction).

e/ Calculating the redness as R/(R+G+B), following the methodology outlined in Bertin et al. (2023).

The detailed steps for image extraction and redness measure are available as supplementary information (S.1).

2.1.4. Statistical analysis

All the analyses were performed with the software R (version 4.1.2, R Core Team, 2021).

For the statistical analysis, the median redness of each facial region per hen and situation was used. As each hen was observed in multiple situations, a linear mixed model was performed. The model included the regions of interest (comb, wattle, cheek, ear lobe), the situations grouped by valence and arousal (positive situations of low arousal, V+/A-; positive situations of high arousal, V+/A+; negative situations of high arousal, V-/A+) and the interaction between both as fixed effects; and the identity of the hen as random effect. The lmer function of the package lme4 was used (Bates et al., 2015). The normality of the residuals and the homogeneity of the variance were confirmed using the package DHARMa (Hartig, 2022). The post-hoc analysis for comparing situations was carried out using the emmeans function of the package emmeans (Lenth, 2022) (p-value adjust by Tukey).

Data are expressed as median [1st quartile - 3rd quartile]. Test significance was considered at $p \leq 0.05$.

2.2. Experiment 2: facial redness and S-IgA concentration as a marker of the human-hen relationship

2.2.1. Animals and housing

26 domestic hens (hybrids between male Sussex and female Label rouge strains; Ferme Avicole du Mont d'Or, 82500 Larrazet, France), were housed in two groups of 13 at INRAE, UE EASM (Le Magneraud, 17700 St Pierre d'Amilly, France). The birds were 49 days of age when they arrived. Each group had access to a 25 m^2 house (5.15 m x 4.85 m) with wooden litter and a 500 m^2 (27 m x 27 m) meadow-like outdoor range with grass. The house was furnished with two heat lamps (17°C), artificial lighting (from 9:00 am to 5:30 pm), a perch, four nests, a feeding trough (diameter: 41 cm) and a drinker (diameter: 32 cm). The outdoor range, covered with wire mesh, was accessible via two trapped doors and was furnished with a 1 m^2 hut. Water and feed were provided *ad libitum*. Before and after the habituation, the hens were housed in individual cages to collect feces (Fig. 2).

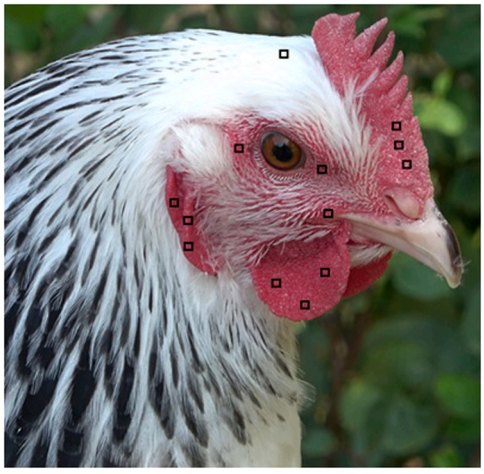

Fig. 1. Position of the 10×10 pixels squares on the comb, cheek, ear lobe, wattle and white feather.

2.2.2. Habituation to human

The hens were divided into two groups (matched for weight) of 13: a group habituated to human interaction (H hens) and a control group non-habituated to human interaction (NH hens). However, at the conclusion of the experiment, it was discovered that one individual from the non-habituated group (NH) exhibited male characteristics. Consequently, this individual was excluded from all analyses, resulting in an effective sample size of n=12 for the NH hens. The habituation procedure started when the hens reached 63 days of age and spanned 5 weeks, continuing between the novel environment and the reactivity to human tests (Fig. 2). This habituation process comprised two daily sessions, one in the morning and one in the afternoon, lasting 2 hours each from Monday to Friday. Within each session, the experimenter adopted a static posture twice, with durations of 30 and 40 minutes, respectively. Subsequently, mealworms paired with a clicker were individually distributed to each hen (three separate sessions of 5 minutes, spaced by 5 minutes – totaling 25 minutes) after each static period. Throughout these sessions, the experimenter engaged with the hens verbally and, if permissible, through gentle physical contact. The habituated group received a higher level of experimenter presence compared to the non-habituated group during the interval between the reactivity to human test and the generalization to human test (Fig. 2). Protocols were in place to ensure a gradual and non-threatening approach, with bird caretakers following practices such as knocking before entering the hen house and avoiding sudden or rapid gestures. For the non-habituated group, mealworms were dispensed from outside the house via a wire pipe to prevent any direct association with the experimenter. Caretakers refrained from knocking before entering the hen house and maintained a neutral demeanor towards the hens, minimizing verbal interaction to create a more neutral environment.

2.2.3. Effect of the habituation to human

2.2.3.1. Effect on behaviors. To evaluate the effect of habituation on the behavior of the hens, the behaviors of each group were compared before and after the habituation, at 59 and 95 days respectively (Fig. 2). The experimenter sat on a chair between the hen house and the outdoor area for 1 hour in the morning and 1 hour in the afternoon. The experimenter noted the hen behaviors and their proximity to the experimenter, by scan sampling (Altmann, 1974), every 2 minutes resulting in 31 scans in the morning and 31 scans in the afternoon. The following behavioral repertoire was used: comfort behavior (preening, dustbathing, scratching, stretching, ruffling, flapping wings), resting, feeding (eating and drinking), locomotion (walk, run, fly) and exploration (pecking and scratching environment or nest, and observing environment by moving the head). The measure of proximity to the experimenter consisted of three categories: close (at a distance inferior to the length of one hen), intermediate (at a distance inferior to 150 cm) and far (at a distance superior to 150 cm).

We also performed a test of generalization at the end of the experiment (116 days) to evaluate the reaction of hens toward different humans (Fig. 2). This test was performed inside the hen house in the morning and in the afternoon. The experimenter who had performed habituation and a caretaker familiar for both H and NH hens sat with their legs extended in front of them and facing each other at around 4 m. Their position was reversed for the second test. During 20 minutes they filmed approaching hens (Sony FDR-AX53 4 K camera). The number of hens that came into physical contact with the experimenter and caretaker and the latency to approach were measured from the films. If a hen did not come into contact then its latency was equal to the test duration

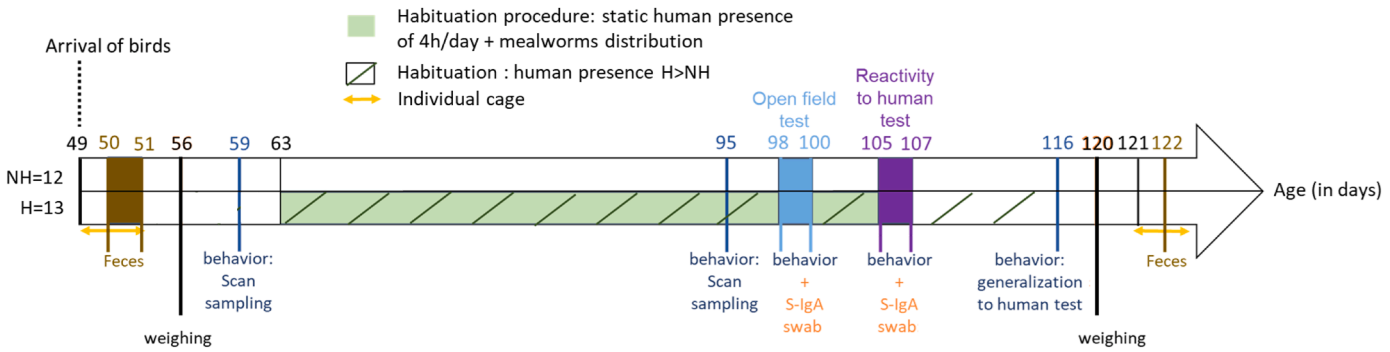

Fig. 2. Time schedule of the experiment. H: Habituated group, NH: Non-habituated group.

i.e. 1200 s.

2.2.3.2. Effect on growth and S-IgA concentration. To evaluate the effect of the habituation on the growth, weight was measured before and after habituation, at 56 and 120 days respectively (Fig. 2).

To test for the cumulative effect of the habituation procedure on the S-IgA concentration, hens were placed in individual cages and fresh feces were collected before and after habituation (Merino-Guzmán et al., 2017) (Fig. 2). Before habituation, 2 days were necessary to collect enough fresh feces but only 1 day after habituation. A volume of 1.5 ml of feces was collected from each hen and stored at −20°C until analysis.

2.2.4. Redness and S-IgA concentration as bio-markers of human-hen relationship

To evaluate the relevance of using the redness of the skin and S-IgA concentration as bio-markers of the human-hen relationship, two behavioral tests were performed. We first tested the general underlying fearfulness of hens in an open field test (Forkman et al., 2007) to control for the effectiveness of the habituation procedure in reducing specifically fear of humans and not other dimensions like fear of novelty or social separation. Then we tested the response to human presence in a reactivity to human test (Waiblinger et al., 2006). The tests were performed in a testing area with opaque walls (length=195 cm, width=105 cm) situated in a corner of the hen house. Natural light was supplemented by artificial lights (OS RAM L36W / 865 lumilux cool dayligt). Hens were filmed (same cameras as in experiment 1) through two trapdoors (length=31 cm, height=21 cm) at hen height: one in the middle of the lateral wall and one in the door in the middle of the width.

2.2.4.1. Behavioral tests. The open field test was conducted following a 5-week habituation period when the hens were between 98 and 100 days old (Fig. 2). Each hen underwent individual testing in the unfamiliar testing area for 15 minutes, a duration chosen based on studies in mammals where the time allowed for S-IgA to respond to the situation ranged from 10 to 15 minutes (Harrison et al., 2000; Hucklebridge et al., 2000; Lv et al., 2018; McCraty et al., 1996). Due to the need for IgA sampling at the end of each test, 3 days were required to test all hens from both groups. Each testing day comprised two sessions: one in the morning and one in the afternoon, with two or three hens from each group tested during each session. To facilitate handling, all hens were gathered in a corner of their hen house at the start of the session. The tested hen was transported in a cardboard box by the experimenter responsible for habituation and placed in the left corner opposite the door. Measured behaviors included the latency before taking the first step, the duration of exploration (time spent pecking or scratching the environment) and locomotion, and the frequency of comfort behaviors (preening, flapping wings, and stretching) and escape attempts (jumps against the wall and actual escape through the lateral trapdoor of the camera). In cases where a hen escaped (H-hen=3, NH-hen=1), she was transported again in the cardboard box and placed in the same corner to achieve a total 15-minute duration in the test area.

The reactivity to human test occurred 1 week after the open field test, when the hens were between 105 and 107 days old (Fig. 2). Hens had at least 4 days to explore the testing area. The door was left open after the last open field test and the feeding trough was placed inside. The procedure mirrored that of the open field test, with the experimenter sitting inside the testing area without moving, back against the door. Two cameras captured the hens: one through the lateral trapdoor and one handheld by the experimenter. Measured behaviors included those from the open field test, along with the latency to make contact with the experimenter, time spent exploring the experimenter (pecking the experimenter or its camera) and time spent in the zone close to the experimenter (half of the testing area where the experimenter was sitting). Procedures for escaped hens remained consistent with the open field test (H-hen=0, NH-hen=2).

2.2.4.2. Measure of facial redness. The method of image analyses was the same as for experiment 1. A total of nine profiles per bird and per test were taken (three per 5 minutes periods), and only one 10×10 pixels square was positioned for the ear lobe. In addition, we controlled the basal level of skin redness with two or three images per hen during one spontaneous resting filmed in the 2 weeks preceding the tests.

2.2.4.3. Secretory IgA sampling. Following each test, all birds underwent mucosal fluid sampling, with collection from the cloaca, saliva, and lachrymal fluid. The cloacal samples involved the application of nylon flocked swabs with a circular movement on the cloacal mucosa for 10 seconds. Salivary swabs were used on the buccal mucosa to collect saliva. The swabs were then placed in 15 ml Eppendorf tubes containing 1 ml of PBS 1X, mixed using a vortex, and stored at −20°C for later analysis. The collection of lachrymal fluid followed the method outlined by Merino-Guzmán et al. (2017) and Toro et al. (1993). Fine sodium chloride crystals were placed at the corner of the eye, and tears were collected at the opposite corner using a micropipette and stored at −20°C for later analysis.

2.2.5. Secretory IgA assays

The commercial Chicken IgA ELISA Kit (ab157691, Abcam) was used according to the manufacturer's instructions. Briefly, IgA present in feces, lachrymal fluid and swab samples reacted with anti-IgA antibodies adsorbed to the surface of polystyrene microtiter wells (Nunc MaxiSorp, Thermo Fisher Scientific). After removing unbound proteins through washing, anti-IgA antibodies conjugated with horseradish peroxidase were added. These enzyme-labeled antibodies formed complexes with the previously bound IgA. Following another washing step, the enzyme bound to the immunosorbent was assayed by adding a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine. The quantity of bound enzyme directly correlated with the concentration of IgA in the sample, and the absorbance at 450 nm served as the measure of IgA concentration. IgA quantity in tested samples was interpolated from the

standard curve constructed from the standards and corrected for sample dilution using Microsoft Excel. Chicken IgA concentration was determined against a standard curve provided with the kit, with optimal dilutions for quantitation set at 1:10,000 for lachrymal fluid, 1:100 for mouth swab, 1:100 for cloacal swab, and 1:1000 for feces.

2.2.6. Statistical analysis

To evaluate the habituation of hens, the behavior of the hens and the proximity to human inside each group were compared before and after habituation with permutation tests for paired data. We used the function symmetry_test of the package coin (Hothorn et al., 2008, 2006). For the generalization to human test, the data from the morning and afternoon were pooled. The number of different hens of each group coming into physical contact with the experimenter and the caretaker during the day was compared using a chi square test, or fisher test if the data size was too small (chisq.test and fisher.test respectively). The mean latency to contact the experimenter and the caretaker during the day was compared between the two groups with permutation tests (oneway_test - package coin). To evaluate the effect of the habituation on individual growth and the cumulative effect on the S-IgA concentration, the two groups were compared with permutation tests (oneway_test - package coin).

For the two behavioral tests, the two groups were compared with permutation tests for behaviors, mean redness and S-IgA concentration (oneway_test - package coin). The same test was used for comparing the mean redness when resting. The number of hens expressing comfort behaviors and escape attempts were compared between the two groups with chi square test, or fisher test if the data size was too small (chisq.test and fisher.test respectively).

Data are expressed as median [1st quartile - 3rd quartile]. Test significance was considered at $p \leq 0.05$.

2.2.7. Ethical note

All experimental procedures were approved by the Ethics Committee Poitou-Charentes n°84 (reference number: APAFIS#27870–2020100 51730296 v5) and carried out following current European legislation (EU Directive 2010/63/EU).

3. Results

3.1. Experiment 1: facial redness in contrasted emotional situations

The linear mixed model shows a significant effect for the three fixed effects: the regions of interest (cheek, ear lobe, wattle, comb; $F(3, 127) = 66.86$, $p<0.001$), the situations grouped by valence and arousal (V+/A-, V+/A+, V-/A+; $F(2, 127) = 141.40$, $p<0.001$) and the interaction between the regions of interest and the grouped situations ($F(6, 127) = 4.98$, $p<0.001$). For the cheek, ear lobe and wattle, V-/A+ was significantly redder than V+/A+ and V+/A+ was significantly redder than V+/A- ($p \leq 0.001$ for all cases; Fig. 3A). For the comb, V-/A+ was not significantly redder than V+/A+ ($p=0.122$) but was significantly redder than V+/A- ($p<0.001$) and V+/A+ tend to be significantly redder than

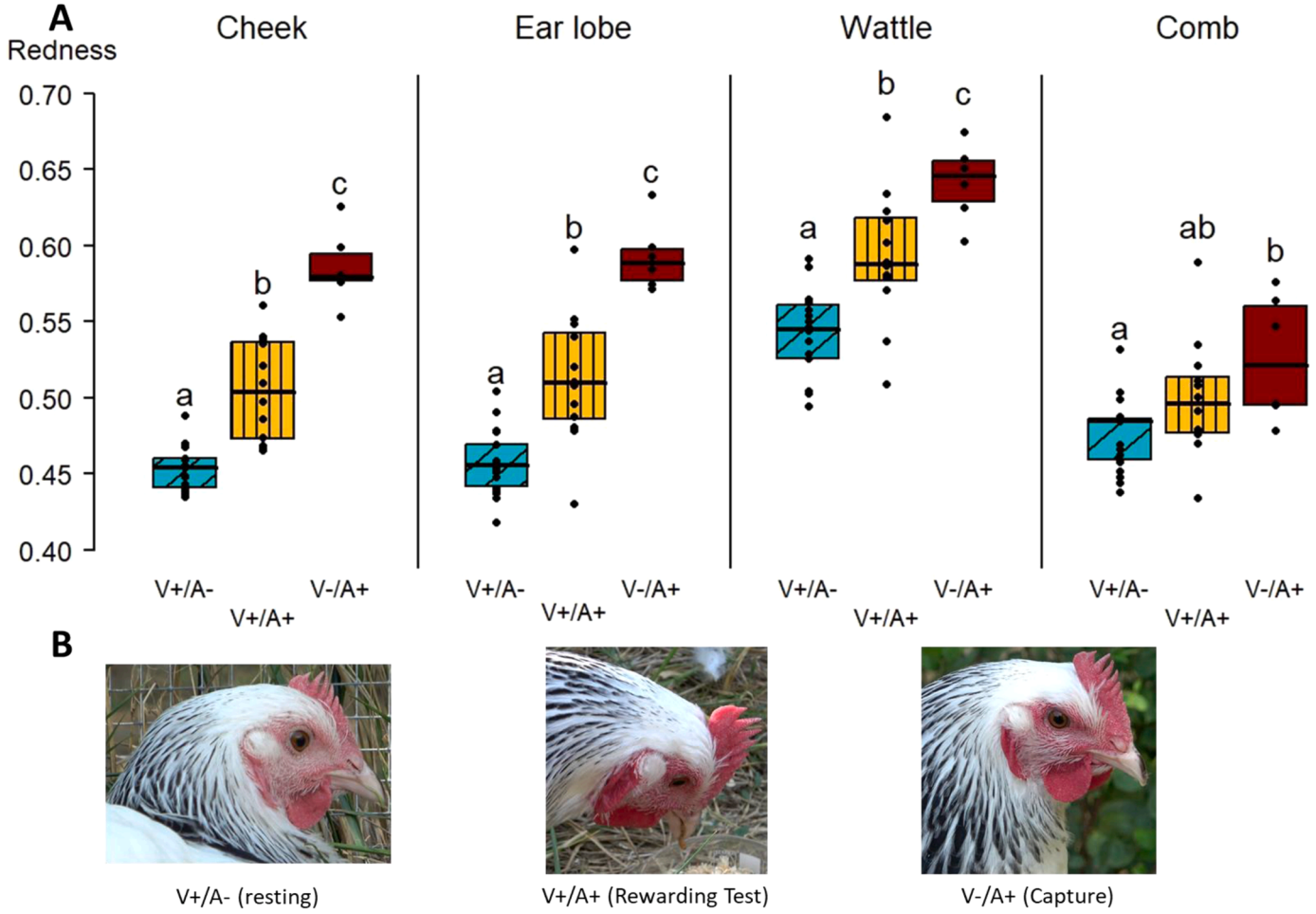

Fig. 3. Redness depending on the situations for the cheek, ear lobe, wattle and comb (A). Illustration of the redness of the same hen in one of each kind of situations (B). V+/A-: situations of positive valence and low arousal; V+/A+: situations of positive valence and high arousal; V-/A+: situations of negative valence and high arousal. Different letters indicate significant differences: $p<0.05$.

V+/A- (p=0.060). Fig. 3B illustrates the skin redness for each of the three situations.

3.2. Experiment 2: facial redness and S-IgA concentrations as a marker of the human-hen relationship

3.2.1. Effect of the habituation to human

3.2.1. .1 Effect on behaviors. After the habituation procedure, both H and NH hens exhibited significant changes in behavior. H and NH hens consumed less feed and explored their surroundings more compared to pre-habituation levels (Table 1). H hens displayed a significant increase in comfort behaviors compared to pre-habituation levels (Table 1). Furthermore, post-habituation, H hens were significantly more observed at close distances and intermediate distances from the experimenter, and showed no significant difference at far distances compared to their pre-habituation behavior. In contrast, NH hens predominantly maintained a distant position from the experimenter, with no significant difference observed before and after habituation (Table 1).

During the generalization to human test, significantly more H than NH hens came into contact with the experimenter (12 out of 13 hens vs 3 out of 12 hens; chi-squared (1)=11.58, p<0.001), and significantly more H than NH hens came into contact with the caretaker (12 out of 13 hens vs 2 out of 12 hens; odds ratio=0.03, p<0.001). The H hens came significantly faster than the NH hens into contact with the experimenter (381 s [187−650] vs 1200 s [1083–1200], Z=-3.54, p<0.001), and with the caretaker (706 s [514−865] vs 1200 s [1200−1200], Z=-3.03, p<0.001).

3.2.1.2. Effect on growth and S-IgA concentrations. The weights of the hens did not differ significantly between H and NH hens before (0.88 kg [0.81–0.90] vs 0.89 kg [0.85–0.93], Z=-1.32, p=0.194) or after (2.24 kg [2.18–2.30] vs 2.32 kg [2.23–2.40], Z=-1.68, p=0.094) habituation.

The S-IgA concentrations in the feces did not differ significantly between H and NH hens before (117 µg/ml [63−190] vs 127 µg/ml [83–157], Z=0.12, p=0.903) or after (25 µg/ml [20–38] vs 23 µg/ml [18–36], Z=0.14, p=0.693) habituation.

3.2.2. Redness and S-IgA concentration as bio-markers of human-hen relationship

3.2.2.1. Behavioral tests. During the open field test, the behaviors did not significantly differ between the two groups (Table 2).

During the reactivity to human test, H hens started moving significantly faster, came faster into contact with the experimenter, explored her longer, and more individuals displayed comfort behaviors and tended to express less escape attempts than NH hens (Table 2).

3.2.2.2. Measure of facial redness. During resting, the redness did not differ significantly between H and NH hens for the cheek (0.40 [0.39–0.41] vs 0.39 [0.38–0.40], Z=0.46, p=0.654), ear lobe (0.42 [0.41–0.43] vs 0.41 [0.40–0.44], Z=0.20, p=0.844), wattle (0.47 [0.45–0.49] vs 0.48 [0.46–0.50], Z=-0.16, p=0.877) or comb (0.44 [0.43–0.45] vs 0.44 [0.42–0.44], Z=0.41, p=0.697).

During the open field test, the redness did not differ significantly between groups for the cheek (Z=-1.54, p=0.126), ear lobe (Z=-1.00, p=0.323), wattle (Z=-1.30, p=0.198) or comb (Z=-1.24, p=0.218) (Fig. 4A).

During the reactivity to human test, H hens had a significantly lower redness than NH hens for the cheek (Z=-3.64, p<0.001), ear lobe (Z=-3.25, p<0.001), and wattle (Z=-2.39, p=0.015) and there was a tendency for the comb (Z=-1.89, p=0.057) (Fig. 4B).

3.2.2.3. Secretory IgA concentrations. After the open field test, S-IgA concentration did not significantly differ between groups in any samples (saliva: Z=-1.32, p=0.193; cloaca: Z=0.44, p=0.677; lachrymal fluid: Z=-0.99, p= 0.405) (Fig. 5A).

After the reactivity to human test, H hens had a significantly lower S-IgA concentration than NH hens in the lachrymal fluid only (Z=-1.77, p=0.047). The S-IgA concentration did not differ significantly in the saliva (Z=0.64, p=0.552) or at the cloaca (Z=-0.08, p=0.941) (Fig. 5B).

4. Discussion

In our study, we observed significant variations in facial redness among hens exposed to contrasting emotional contexts in a semi-natural setting in the first experiment. Notably, hens exhibited less redness in calm situations compared to rewarding situations, while they appeared reddest in fear-related situations. In the second experiment, H hens displayed lower redness when tested alone with a human than NH hens. This outcome suggests that variations in facial redness could serve as an indicator of how animals perceive specific stimuli in their environment. While short-term variations in S-IgA concentration were observed in the lachrymal fluid, further studies are necessary to evaluate the relevance of this marker.

In the first experiment, significant variations in skin redness were observed across all studied regions (cheek, ear lobe, wattle, and comb). For all regions except the comb, hens exhibited the highest skin redness in negative situations of high arousal associated with fear, followed by positive situations of high arousal associated with reward acquisition, and the lowest skin redness in positive situations of low arousal associated with calm and contentment. For the comb, hens exhibited significantly more skin redness in negative situations of high arousal than in positive situations of low arousal. The skin redness during positive situations of high arousal fell between the two. These skin redness patterns observed in juvenile Sussex hens align closely with results found in the cheek and ear lobe regions of two other juvenile hen strains (Pekin and Meusienne) in a similar study (Arnould et al. in revision). The comb region in Sussex differed from the other facial regions, showing a response not significantly different in skin redness for positive situations

Table 1
Behavioral observations for each group of hens before and after the habituation procedure.

	Habituated hens				*Non-habituated hens*			
	Before habituation	After habituation	Z*	p-value*	Before habituation	After habituation	Z*	p-value*
comfort	**7 [6–8]**	**12 [10–14]**	**-2.62**	**0.003**	7 [5–11]	9 [6–10]	-0.35	0.790
resting	21 [20–23]	19 [17–21]	1.07	0.327	19 [15–20]	24 [18–26]	-1.45	0.162
feeding	**14 [11–17]**	**6 [6–7]**	**3.04**	**<0.001**	**20 [15–23]**	**7 [5–10]**	**3.04**	**<0.001**
locomotion	4 [3–5]	6 [4–8]	-1.00	0.364	5 [5–7]	9 [6–10]	-1.89	0.057
exploration	**14 [13–18]**	**17 [12–22]**	**-2.08**	**0.027**	**12 [8–14]**	**15 [14–16]**	**-2.37**	**0.007**
distance close	**0 [0–2]**	**6 [3–10]**	**-2.12**	**0.025**	0 [0–0]	0 [0–0]	-	-
distance intermediate	**41 [36–47]**	**38 [32–42]**	**1.96**	**0.047**	24 [21–28]	23 [18–24]	1.73	0.097
distance far	17 [13–25]	16 [10–25]	0.53	0.666	39 [34–42]	40 [38–44]	-1.51	0.149

Data are presented as median and quartiles (median [1st quartile - 3rd quartile]) of the number of times a behavior was observed and of the number of times hens were observed at one of the three distances from human during the 62 scans.

* results of the permutation tests for paired data. Significant results are bolded.

Table 2
Behaviors of habituated and non-habituated hens during the open field test and the reactivity to human test.

	Open field				*Reactivity to human*			
	H hens	NH hens	test statistic*	p-value *	H hens	NH hens	test statistic*	p-value *
Latency before taking the first step	16 [1–47]	40 [16–67]	Z=-1.03	0.419	**11 [4–17]**	**36 [12–102]**	**Z=-2.07**	**0.027**
Exploration	66 [55–100]	28 [6–100]	Z=-0.16	0.912	91 [67−210]	48 [38−344]	Z=-0.44	0.672
Locomotion	259 [151−290]	175 [103−328]	Z=0.46	0.654	176 [106−234]	272 [148−325]	Z=-1.11	0.272
Escape attempts[1]	9/13	5/12	Chi-squared (1)= 0.97	0.325	3/13	8/12	Chi-squared (1)= 3.21	0.073
Comfort behaviors[1]	8/13	3/12	Chi-squared (1)= 2.06	0.151	**8/13**	**1/12**	**odds ratio=15.45**	**0.011**
Latency to contact the experimenter	-	-	-	-	**102 [29–122]**	**156 [94−523]**	**Z=-2.04**	**0.029**
Exploration of the experimenter	-	-	-	-	**10 [6–18]**	**0 [0–6]**	**Z=2.48**	**<0.001**
Time passed in zone close to the experimenter	-	-	-	-	568 [473−687]	521 [305−676]	Z=1.25	0.221

Data, in seconds, are presented as median and quartiles (median [1st quartile - 3rd quartile]). 1: data are presented as the number of hens that displayed the behavior on the total number of hens.
* results of permutation test, chi square test and fisher test. Significant results are bolded.

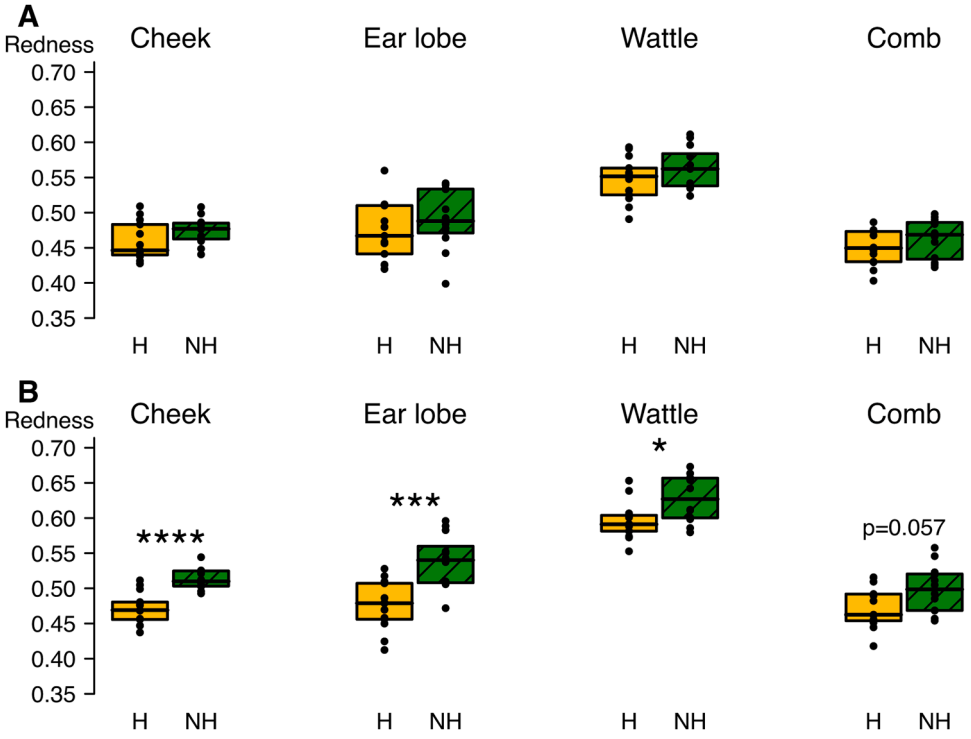

Fig. 4. Redness of the cheek, ear lobe, wattle and comb of the habituated (H) and non-habituated (NH) hens during the open field test (A) and the reactivity to human test (B). Permutation test: *p<0.05, ***p<0.001, ****p<0.0001.

of high arousal compared to the other situations, but tending to be significantly redder than in positive situations of low arousal. The lower response of the comb to different affective states may be attributed to the role of its color after maturation, where it becomes redder and increases in size. For example, the comb size and color informed on the dominance relationship in hens (O'Connor et al., 2011). Having other roles, comb variations in coloration associated with affective states may be less pronounced. Nonetheless, despite the small sample size in our study, the consistent results across the three observed strains strengthen our findings. In summary, less red facial skin is observed in situations of calm and contentment.

States of calm and contentment in hens may be associated with the activity of the parasympathetic system, as proposed by Richardson et al. (2016). According to their model, affective states result from the inter-regulation of three systems. The drive system, linked to the activation of the reward acquisition system and involving dopamine, corresponds to positively valenced and high arousal affective states (V+/A+). The threat system, linked to the activation of the punishment avoidance system and involving adrenaline, cortisol, and noradrenaline, corresponds to negatively valenced and high arousal affective states (V-/A+). The contentment system, associated with the turn off of the punishment avoidance and reward acquisition systems and involving the oxytocin and opiate system, corresponds to positively valenced and low arousal affective states (V+/A-). Increases in skin redness might be induced by the activity of the sympathetic system occurring in high arousal situations, while low skin redness observed in contentment situations might result from the activity of the parasympathetic system turning off the drive and threat systems. Birds known to blush have highly vascularized bare skin, making the face a possible specific target for the autonomic nervous system (Negro et al., 2006).

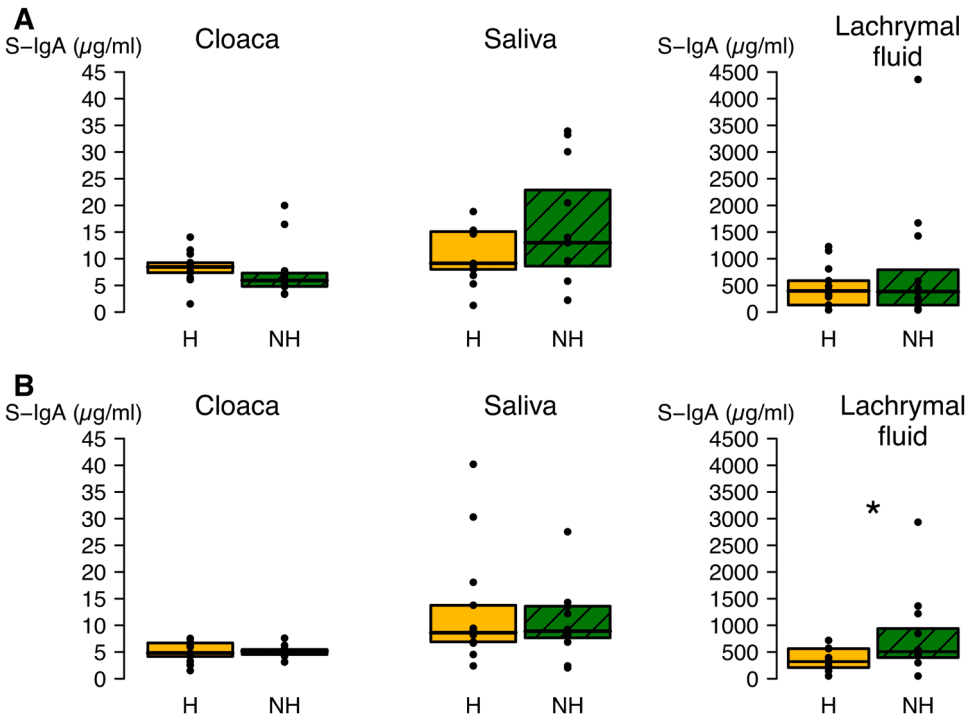

Fig. 5. S-IgA concentration in fluids of the cloacal mucosa, saliva and lachrymal fluid of the habituated (H) and non-habituated (NH) hens for the open field test (A) and the reactivity to human test (B). Permutation test: *p<0.05.

As low skin redness indicates contentment and calmness, skin redness emerges as a potential marker of positive affect. In the context of human-animal relationships (second experiment), we tested this alongside another potential marker of positive affect: S-IgA. Habituation to humans in the H group was confirmed by comparing behaviors before and after the procedure. In contrast to the NH group, the H group expressed more comfort behaviors and was observed closer to the experimenter after habituation. These results align with the literature where an increase in comfort behaviors and a reduction in avoidance behaviors were observed in hens or quails exposed to habituation procedures (Bertin et al., 2019, 2008; Graml et al., 2008). Comfort behaviors are associated with positive affective states (Papageorgiou et al., 2023; Zimmerman et al., 2011) and a close distance to humans is commonly used to infer a good human-animal relationship (Waiblinger et al., 2006). The behavior of H and NH hens also diverged significantly during the generalization test, confirming that H hens have a more positive perception of humans. Almost all H hens came into contact with the experimenter and the caretaker while only few NH hens did. Both H and NH hens ate less and explored more after the habituation procedure, potentially linked to the growth of the birds, which was unaffected by the procedure.

For the behavioral tests, no significant difference in any behavioral parameter was observed between H and NH hens during the open field test used for testing general underlying fearfulness, whereas their behavior diverged significantly during the reactivity to human test. In the open field test, hens of both groups stayed immobile before starting to move, which is a fear-related behavior (Forkman et al., 2007; Jones, 1996). They also attempted to escape the testing arena, which can be a marker of both fear and social reinstatement behaviors, as the bird are isolated (Forkman et al., 2007). In presence of the experimenter (reactivity to human test), H hens started moving faster and showed higher number of comfort behaviors, faster contact with the experimenter and longer exploration of the experimenter than NH hens. These behavioral parameters indicate that H hens expressed less fear-related behaviors than NH hens and that the experimenter might have been a positive stimulus, as they came to explore them. Our data are in accordance with the literature on domestic fowl showing that procedures of habituation to human reduce specifically the fear toward human and not the general underlying fearfulness (Bertin et al., 2019).

Concerning skin redness, the habituation procedure did not affect its basal level measured during resting. The skin redness of the hens did not differ in the open field test aligning with the behavioral results. In contrast, during the reactivity to human test, the H hens had a lower redness than the NH hens for the cheek, ear lobes, wattles and this was a tendency for the comb. In accordance with the results of our first experiment and those obtain on two other strains of hens (Arnould et al. in revision), the lower redness observed in the H hens suggests less fear. Likewise, blue and yellow macaws and hooded vultures were also less red in calm situations (Bertin et al., 2018; Negro et al., 2006). The cheek and ear lobes better discriminate between the two groups, as the differences were more pronounced, compared to the wattles and comb. However, further experiments are needed to better understand how each region of the hen face varies as a function of the affective states. Nonetheless, the redness of the cheek and ear lobe seem to be a valid marker of the human-hen relationship: a low redness indicating less fearful hens and possibly a more positive perception of human.

Regarding S-IgA, concentrations in cloacal, saliva, and lachrymal fluids did not differ between H and NH hens during the open field test, aligning with behavioral and skin redness results. In the reactivity to human test, only lachrymal fluid S-IgA concentration differed between the groups. Saliva is commonly used for testing short-term effects of S-IgA in mammals (Staley et al., 2018). Controversial results exist in the literature on salivary S-IgA variation with affective states. Our findings are in accordance with the absence of differences in human salivary S-IgA when recalling happy or guilty memories (Hucklebridge et al., 2000). Conversely, McCraty et al. (1996) observed increased S-IgA when individuals thought of someone they appreciated. Calves also showed higher S-IgA following positive events and lower concentrations after negative events (Lv et al., 2018). Cloacal and lachrymal sample analyses were exploratory, as S-IgA are not typically measured in these mucosae. Our preliminary results showed no difference in cloacal S-IgA between groups. However, in the reactivity to human test, lachrymal fluid S-IgA

concentrations were increased in NH hens compared to H hens. This may be explained by an acute stress response, analogous to dogs experiencing increased lachrymal caruncle temperature during stress, indicating autonomic nervous system activation and enhanced blood circulation in the region (Casas-Alvarado et al., 2022). A similar mechanism in poultry could involve acute stress triggering S-IgA production in the lachrymal gland by increasing blood circulation. At the end of the study, fecal S-IgA concentrations did not differ between groups. Higher S-IgA levels in mammals may be associated with positive situations (Gourkow and Phillips, 2016; Staley et al., 2018), but recent poultry studies found decreased fecal S-IgA in negative housing conditions without variation in positive conditions (Campbell et al., 2023, 2022). Our results suggest that S-IgA concentration may not be a valid marker for the human-hen relationship in our study probably due to favorable housing conditions for both groups.

The present study suggests that a hen's facial redness changes based on its affective state, providing a potential way to assess its well-being and relationship with humans. Although our findings are preliminary due to a small sample size, they hint that less redness in the cheek and ear lobes may indicate calm and contentment states. This introduces the idea of using redness as a welfare marker, but further research is needed. Understanding that the intensity of redness can show how sensitive hens are to different situations and analyzing these variations could help us to grasp their perceptions of the environment. While our study did not find S-IgA concentration as a sensitive marker for assessing the human-hen relationship, the intriguing results in lachrymal fluids suggest more research is warranted in poultry. To sum up, our study sheds light on using facial redness as a potential indicator of avian affective states and human-hen relationship.

CRediT authorship contribution statement

Conceptualization: RG, LL, PQ, KG, FL, SAL, AB, CA; Data Curation: DS, RG, MCB, BP, AB, CA; Formal Analysis: DS, AJ, RG, MCB, BP, GL, AB, CA; Funding Acquisition: RG, LL, PQ, KG, FL, SAL, AB, CA; Investigation: DS, AJ, RG, MCB, BP, VG, FL, SAL, AB, CA; Methodology: AJ, RG, LL, MCB, BP, GL, VG, PQ, KG, FL, SAL, AB, CA; Project Administration: AJ, RG, PQ, KG, AB, CA; Resources: DS, RG, MCB, BP, GL, KG, AB, CA; Software: DS, MCB, BP, GL, AB, CA; Supervision: RG, KG, AB, CA; Validation: DS, AJ, RG, MCB, BP, GL, VG, AB, CA; Visualization: DS, AJ, AB, CA; Writing – Original Draft Preparation: DS, RG, AB, CA; Writing – Review & Editing: DS, AJ, RG, LL, MCB, BP, GL, VG, PQ, KG, FL, SAL, AB, CA

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.applanim.2024.106268.

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