# Development of practical measures in hen welfare

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# Foreword

Public opinion, negative press coverage and concerted efforts by interested parties have pushed animal welfare in the poultry industry to the fore. Whilst considerable efforts have been made to improve animal welfare standards within the sector a fundamental problem remains, namely, how welfare in chickens is measured. Current methods based on hormonal and behavioural measures are expensive, technically demanding and subject to personal interpretation. MicroRNAs are small molecules found in practically all biological fluids in animals and many specific microRNAs increase or decrease in abundance in response to a raft of stimuli, including: pain, hunger, heat exposure, thirst and stress. The responsiveness of microRNAs to these, and other, stimuli makes them an ideal candidate as an objective measure of numerous aspects of animal welfare. Here we have explored microRNAs as measures of both positive and negative affective states in poultry.

Exposure to prolonged stress has detrimental long-term impacts on layer health and that of their progeny. This project aims to establish if layer hens experiencing a negative affective state transfer a stress miRNA profile in their eggs. Layer welfare is centred not only on the absence of stress (negative affective state), but also the presence of enriching emotional experiences (positive affective state). Although behavioural indicators are often used to identify positive affective state, little research has been conducted around what constitutes a positive affective state profile. This project aims to elucidate a plasma miRNA profile indicative of hens experiencing a positive affective state.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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# About the Authors

Associate Professor Tamsyn Crowley is a molecular biologist who has been associated with the poultry industry for 20 years. She is passionate about applying science in industry, and currently holds positions at both Deakin University and the University of New England.

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# Abbreviations

| | |
|---|---|
| cDNA | Complementary DNA |
| Ct | Cycle threshold |
| DS | Difference Score |
| FC | Foraging control |
| FN | Foraging Nalmefene |
| Log-ratio | Logarithmically transformed ratios |
| MC | Mealworm control |
| MN | Mealworm Nalmefene |
| miRNA | Micro Ribonucleic Acid |
| mRNA | Messenger RNA |
| NAS | Negative affective state |
| NTC | No template control |
| PAS | Positive affective state |
| RNA | Ribonucleic Acid |
| RT-qPCR | Reverse Transcription/Real-time Quantitative Polymerase Chain Reaction |
| TI | Tonic immobility |

# Executive Summary

Layer hen welfare is influenced by environmental, social and genetic factors. Good welfare is vital to productivity as stressed hens lay less eggs. Consumers are increasingly demanding that their eggs originate from farms supporting good layer welfare. Moreover, they are also concerned with health implications linked to transmission of stress from hens to eggs, and ultimately the consumer. Hen welfare relies on the absence of stress but also inclusion of rewarding activities. Welfare is best assessed by the constructs of negative and positive affective states. Experiences that reinforce these cognitive pathways contribute to future behaviour, immunity and health. Layers exposed to sustained stress have poorer long-term health outcomes and these negative health attributes can also be transferred through the eggs to their progeny. Developing a method to objectively measure layer welfare would benefit the Australian layer industry and help build consumer confidence around welfare standards. This project aimed to identify blood and egg yolk biomarkers capable of ascertaining the affective state of layer populations. The biomarkers chosen were microRNAs (miRNAs). miRNAs are small RNA sequences that play important regulatory roles in many biological processes through gene silencing. They were selected for this project because they demonstrated differential expression when exposed to environmental and physical stimuli, are relatively conserved across species and retain stability in biofluids. We hypothesised that a hen experiencing a negative affective state will exhibit a different miRNA profile than a hen experiencing a positive affective state.

Negative affective state was induced in hens through social stress associated with changing cage-mates every day for 10 days. Control hens were used for comparison but retained the same cage-mate for the duration of the trial. Plasma was collected from each hen prior to mixing (baseline) and on day 10 of the trial. Eggs were collected 2 days prior to mixing (baseline) and daily until day 9. A positive affective state was induced via a food (mealworm) and non-food (foraging tray) reward, two desirable interactions for hens. Attainment of a positive affective state involves a 'liking' component facilitated by opioid pathways. Injection of an opioid antagonist, Nalmefene, was used to block the reward pathway thus diminishing the chances of treated hens experiencing a positive affective state. Behaviours of treated hens were compared to control hens who did not receive the Nalmefene and were likely to experience a positive affective state as they interacted with either the mealworm or foraging tray. Food and non-food rewards were incorporated to ensure miRNA expression changes were not an artefact of mealworm consumption.

Control hens from the negative affective state trial were used to construct an average non-stressed miRNA profile using 5 target miRNAs by RT-qPCR analysis. This represented biological variability present in a normal healthy layer population. Using this background for comparison, blind samples were analysed against the same 5 target miRNAs to determine their origin as stressed or healthy layer populations. Healthy samples were expected to exhibit similar miRNA expression to controls whilst stressed samples would show divergence (this is captured by a single number known as the Difference Score). An additional aim of this trial was to define the stress miRNA profile detected in eggs laid by hens exposed to chronic stress. Egg yolk samples were sent for genomic sequencing to annotate this profile. Following this we were able to interrogate the egg samples and indeed detect a profile in eggs.

Control hens from the positive affective state trial interacted more with both the mealworm and foraging tray rewards. Administration of the Nalmefene successfully blocked the reward pathway and reduced anticipatory behaviour in the treated hens. Samples from each of the control and treatment groups were sent for genomic testing to ascertain the miRNA profile of a positive affective state. This research has made a huge leap in terms of knowledge and resources to investigate positive affective states in poultry through the establishment of the first effective animal model and identification of a preliminary biomarker profile.

# Overall Conclusions

This research has successfully met both aims: (1) the development of a non-invasive stress biomarker test; and (2) the ability to measure different affective states in laying hens. It has provided the industry with evidence-based, non-invasive tools for assessing and measuring hen welfare. While the tests are still in their infancy, we have demonstrated that they indeed work and will have future applicability in industry. We have confirmed that it is possible to use eggs, specifically yolks to determine the stress levels in hens. Further we have developed this test to work in pooled samples using RT-qPCR, providing a more viable and cost-effective test for industry as opposed to individual sequenced samples. While we acknowledge that this test is not industry-ready just yet, we are confident that we have laid the foundations and with further industry testing we will have delivered a viable and effective stress biomarker test. This research has for the first time demonstrated that we can illicit and test positive affective states in hens. This ground breaking research has developed and validated the tools required to test for positive affective state in hens, and has identified a preliminary miRNA profile in plasma.

These tools have the potential to enable the broader community to obtain an understanding of the current affective state of welfare of laying hens across all commercially relevant production systems. The Australian poultry industry, namely chicken egg producers, is the main beneficiary of these project outcomes. These evidence-based tests, if adopted by the industry, would provide a means for accurately determining good and poor welfare status within a flock. This is crucial to the industry as it is continually questioned and tested with regard to the status of hen welfare in its production systems.

Currently, many of the large egg buyers (supermarkets) are setting guidelines on how eggs are to be produced in terms theoretically based on the welfare state of layers, but with no means of quantitative measurement. While good welfare of layers is the goal for both producers and consumers, many of the guidelines presently being formulated are based on consumer perceptions rather than facts. The tests developed in this project seek to provide industry with reliable and simple tests that the industry can use to monitor the welfare status of production systems, and which will enable it to make informed decisions about management changes. This project provides tools that will have a significant impact in social terms, as the industry will be able to directly report on the welfare status of all poultry egg production systems.

# 1 Background

Heavily driven by public perception and large supermarket monopolies, welfare is of major concern for the poultry industry. The past few decades have seen the development of poultry practices with a heavy focus on the welfare of poultry. There have been huge changes implemented to accommodate improvements in welfare particularly in the layer industry. There are now a number of production systems including caged and free range, which enable consumers to exercise a conscious choice about how their eggs have been produced. These advances are closely regulated in most countries with regular inspections and audits to ensure that the industry is committed to improving welfare standards. While these developments have undoubtedly facilitated an increase in perceived welfare standards, there is little research to confirm that there has been an actual increase in welfare standards and reduced stress on the birds. Many of the demands placed on the industry in terms of welfare are essentially based on public perception and interpretation, thus making it difficult to objectively assess the real welfare situation. Currently there are a number of behavioural and other tests (corticosterone levels) that have provided an insight into the welfare of poultry.

Assessment of animal welfare is often an attempt to evaluate affective states. Another way to describe this would be that animal welfare is an attempt to understand how an animal perceives its environment or events in its environment. Therefore, there is a need to develop biomarkers for positive welfare states like reward and contentment, and biomarkers for negative welfare states like pain, anxiety and distress. Many of the existing methods used to assess animal welfare such as activation of the hypothalamo-pituitary adrenal axis, glucocorticoid and catecholamine production, increased heart rate and heart rate variability, are as likely to indicate excitement and reward or positive welfare as they are to indicate fear and anxiety or compromised welfare. A novel biomarker of animal welfare should therefore enhance our ability to assess the welfare of animals in situ, should fit within the flow of production, and should be objective and repeatable whilst allowing producers to independently assess the welfare of their animals. This project will enable the layer industry to provide accurate scientific information on the welfare status of their production systems and enable auditors to directly test welfare during the course of their routine inspections.

## 1.1 Hen layer welfare – affective state

Hen layer welfare comprises complex interaction of genetics, physical and social variables, making objective assessment difficult and standardisation of welfare indicators problematic (Hemsworth 2021). Australian consumers often scrutinise layer welfare, attaching personal values to production systems. Free range eggs are perceived as natural whilst caged eggs are associated with layer stress (Bray & Ankeny 2017). This contradicts behavioural and physiological welfare markers indicating hens can experience both stress and good welfare across all production systems (Hemsworth 2021). Animal welfare theories centre on the concept that animals experience positive and negative affective states shaped by exposure to environmental rewards or punishers (Deakin et al. 2016). An animal's long-term affective state, and thus welfare, is a construct of the balance between positive and negative experiences in day-to-day life. Moreover, welfare is not determined simply by the absence of stress but also by the presence of enrichments capable of inducing a reward state (Taylor et al. 2020). Hens are highly motivated to perform certain activities (foraging, dustbathing) that they find rewarding and which, in turn, reinforce behaviours indicative of a positive affective state (Hemsworth 2021). In contrast, exposure to prolonged stress causes deleterious changes in animal cognition, immunity and welfare (Downing & Bryden 2002; Hemsworth 2021). This has long-term welfare impacts as animals demonstrate judgement or cognitive bias (similar to optimistic or pessimistic traits in humans) based on affective state, which can reinforce future behaviour in a negative direction (Deakin et al. 2016).

## 1.2 Current welfare indicators

Current indicators of hen welfare include behavioural measures (feather loss, excessive pecking) and biological measures (corticosterone, adrenalin, heterophil:lymphocyte ratio) (Wade et al. 2018; Hemsworth 2021). Accurate measurement of plasma stress hormones can be compromised due to fluctuations from handling and diurnal variations, but slower formation of eggs (approximately 6 hours) may provide a clearer indication of a hen's stress state (Downing & Bryden 2002). Methods for objectively measuring affective state offer the layer industry a tool to assess animal welfare (Deakin et al. 2016). This has implications for layer management since hen welfare and productivity are inextricably linked. Layers with prolonged elevations in corticosterone resulting from exposure to chronic stress have reduced egg production (Forder 2021; Downing & Bryden 2002).

## 1.3 Novel biomarkers of affective state – microRNA

MicroRNAs (miRNAs) are small RNA sequences that play important regulatory roles in many biological processes through gene silencing (Hicks et al. 2008). One miRNA has the potential to regulate hundreds of messenger RNA (mRNA) making them an important component of biological control systems in all living organisms (Jonas & Izaurralde 2015). The significance of miRNA gene regulation has been demonstrated in various physiological pathways across multiple species (Bartel 2004). They were selected for this project because they show differential expression when exposed to environmental and physical stimuli and retain stability in biofluids (Harrill et al. 2016).

## 1.4 Stress transmission through eggs

Along with nutrients for growth, bioactive molecules including miRNAs are packaged in chicken eggs contributing to epigenetic programming and development of the chick (Wade et al. 2016). miRNA expression is altered through exposure to environmental challenges inducing stress (Harrill et al. 2016). These alterations can be studied to detect patterns of miRNA expression representative of a hen's affective state. Maternal hen exposure to environmental stressors has been shown to affect long-term growth, immunity and behaviour in progeny (Forder 2021). As such, it can be surmised that a hen exposed to stress will lay an egg exhibiting an miRNA profile exerting regulatory effects indicative of a negative affective state on the developing chick or potentially the human consumer. It has been noted by Bray and Ankeny (2017) that consumers are concerned that by eating eggs from a stressed chicken they are ingesting the stress transmitted from the hen.

## 1.5 Project aims

The two project objectives were to:

- further develop an existing stress assay so that it can be applied non-invasively using eggs as the source of miRNAs; and
- identify miRNAs that can identify negative and positive affective (emotional) states in layer chickens.

## 1.6 Project design

Chronic stress or negative affective state was induced in laying hens by mixing them with an unfamiliar cage-mate each day for 10 days. Induction of a negative affective state was validated through observation of behavioural measures. Previous genomic sequencing results provided miRNA candidates indicative of a negative affective state. Five miRNAs previously identified in a Poultry CRC

were selected as the basis of a diagnostic assay allowing comparison of miRNA profiles from different sample populations. Relative expression of the 5 target miRNAs was used to calculate a single digit output known as a Difference Score. The Difference Score value indicated if a sample originated from a stressed or healthy hen population (Wade et al. 2018). Egg yolks laid by negative affective state hens were sequenced to establish if similar stress miRNA profiles are packaged into eggs.

We sought to determine if a positive affective state or reward miRNA profile is expressed in plasma of laying hens. This would enable objective differentiation between hens truly in a positive affective state, versus hens assumed to have good welfare merely due to the absence of stress. Positive affective state was induced by food (mealworm) and non-food (foraging tray) rewards. Both were incorporated in project design to account for potential miRNA alterations resulting from consumption of the reward meal itself. Treatment with an opioid antagonist (Nalmefene) blocked reward pathways by reducing motivation to engage with the rewards. Comparison between treated and non-treated groups established a specific positive affective state miRNA expression profile. Control and Nalmefene hens were exposed to the same external influences. As such, alterations in miRNA profiles between groups can be deemed to originate purely from alterations in cognitive or reward pathways (moods).

## 1.7 What we know, what we don't know

This project furthers research by Wade et al. (2016) who were the first to successfully isolate miRNAs from chicken egg albumin and yolk. Results from genomic sequencing from chronically stressed hens carried out by this group was used to select 5 target miRNAs as the base of the current assay. To the best of our knowledge, we are the first to identify a distinct stress profile in chicken plasma and egg yolk. This project builds on work by Fountain et al. (2020) and Taylor et al. (2020) who induced reduced anticipatory behaviours and operant learning in chickens by blocking reward pathways with Nalmefene administration. To our knowledge, this is the first time that the miRNA profile of a positive affective state chicken has been detected.

# 2 Methodology

## 2.1 Negative affective state

### 2.1.1 Animal housing

Isa brown free range hens (n = 80) at 17 weeks of age were sourced from a commercial pullet farm and transported to a naturally ventilated poultry facility at the University of New England (Armidale, NSW). Hens were vaccinated before arrival and beak trimmed at day old. Hens were housed in pairs in a cage (50 x 54 cm). Hens were provided with a commercially available coarse layer mash (Norco, South Lismore, NSW) ab libitum and water was available at all times via nipple drinkers. Hens were given eight weeks to habituate to the new housing, people and feed. Treatments and sample collection began when hens were 24 weeks of age.

### 2.1.2 Chronic stress treatment

Hens from the chronic stress treatment group (n = 40) were mixed with an unfamiliar hen each daily for a period of ten days. Half of the hens were moved to a new cage every second day, thus although each day hens were introduced to an unfamiliar hen, each hen was moved only every second day, and for a total of five times. During the mixing period, hens within the stress treatment group had food access restricted, to share one feeding tray between two cages (four hens) (Figure 1). This resulted in only one hen from each cage being able to access feed at the one time, resulting in competition for resources with the aim of increasing aggressive interactions and thus chronic stress. Hens from the control group were never mixed during this period, remained undisturbed except for feeding and visual health checks each day, and had a feed trough for each cage that was long enough and contained enough feed so that there was adequate space and feed for two hens to feed at the same time, minimising the chance of competition between the two hens for feed.

**Figure 1 Cage design for negative affective state trial**

Note only 1 feeding trough per 4 hens, designed to increase social stress.

### 2.1.3 Behavioural indicators of stress

A state of tonic immobility (TI) is an antipredator response, and therefore likely reflects fearfulness related to predation, handling and social isolation, but it has also shown to be reflective of general fearfulness (Broom & Johnson 1993). Hens (n = 40 control; n = 40 treatment) were walked to a quiet room adjacent to the housing (< 20m) for testing. Each hen was inverted and restrained gently on its back in a U-shaped cradle with light pressure applied to the sternum, and the head was lightly covered by the handler for 15 seconds. A maximum of three attempts were made to induce the TI state. A successful induction was considered when the chicken remained in TI for more than 15 seconds after the handler released pressure. The length of time chickens remained in TI was recorded. Chickens were permitted to remain in a TI state for a maximum of 600 seconds, after which they were gently righted. If TI was not induced after three attempts, that chicken was given a score of zero.

### 2.1.4 Blood sample collection

Blood samples (4 ml) were collected from all hens, from the brachial vein with a 23-guage needle. Samples were collected into a syringe and immediately transferred into an EDTA Vacutainer (BD Vacutainer, USA) and mixed 180 degrees slowly 10 times, then stored on ice (4°C) for later processing at the laboratory; but were left for a minimum of 1 hour, but no longer than 4 hours. Samples were centrifuged at 1300G for 10 minutes at 4°C and supernatant was collected. Further centrifugation at 3000G for 10 minutes at 4°C ensured that all platelets were removed. Samples were stored at -20°C and transported on dry ice to Deakin University for analysis.

### 2.1.5 Egg sample collection

Eggs were collected and sampled daily from two days prior to treatment (e.g. baseline samples) until two days after treatment. Egg yolk and albumin were collected separately into PCR microtubes (Maxymum recovery, Axygen, Corning, NY, USA) and stored at -20°C before transporting on dry ice to Deakin University for analysis.

## 2.2 Positive affective state

### 2.2.1 Animal housing

Isa brown free range hens (n = 80) at 17 weeks of age were sourced from a commercial pullet farm and transported to a naturally ventilated poultry facility at the University of New England (Armidale, NSW). Hens were vaccinated before arrival and beak trimmed at day old. Hens were housed in pairs in a cage (50 x 54 cm). Hens were provided with a commercially available coarse layer mash (Norco, South Lismore, NSW) ab libitum and water was available at all times via nipple drinkers. Hens were given three weeks to habituate to the new housing, people and feed. Treatments and sample collection began when hens were 19.5 weeks of age.

### 2.2.2 Treatment

The objective was to induce a state of reward and a positive affective state, and to validate with both food and non-food models. Two reward treatment groups were chosen to identify any differences due to differences in consumption of feed rewards (e.g. those that received the drug may not eat the mealworms), and those experiencing reward when provided with a non-consummatory reward. Hens

were randomly allocated to either a food reward or foraging tray reward group, and further randomly distributed into either treatment or control (Table 1).

**Table 1 Positive affective state treatment groups, investigating differences between food or foraging rewards using opioid antagonist Nalmefene**

| Treatment group | Treatment description |
|---|---|
| Food Reward Control **(FRC)** n = 25 | Control hens received a 0.5 ml injection of saline 30 minutes before being offered a food reward (mealworms) |
| Food Reward Treatment **(FRT)** n = 25 | Treatment hens received a 0.5 ml injection of Nalmefene (0.4 mg/kg) 30 minutes before being offered a food reward (mealworms) |
| Foraging Tray Control **(FTC)** n = 25 | Control hens received a 0.5 ml injection of saline 30 minutes before being offered a foraging tray for 10 minutes |
| Foraging Tray Treatment **(FTT)** n = 25 | Treatment hens received a 0.5 ml injection of Nalmefene (0.4 mg/kg) 30 minutes before being offered a foraging tray for 10 minutes |

Treatment hens from both the food reward (FRT) and foraging tray (FTT) groups were dosed with 0.5 ml of Nalmefene (17-[cyclopropylmethyl]-4,5α-epoxy-6-methylenemorphinan-3,14-diol, Nalmefene hydrochloride, 1B/220482, Tocris, Noble park, Victoria, Australia) dissolved in 0.9% saline in the morning, administered via intramuscular injections into the pectoral muscle. Hens were dosed twice daily; 0.4 mg/kg in the morning and 0.2 mg/kg 12 hours later in the evening. Control hens from the food reward (FRC) and foraging tray (FTC) groups were injected with 0.5 ml of 0.9% saline in the morning and evening, administered via intramuscular injections into the pectoral muscle.

### 2.2.3 Food reward

Exactly 30 minutes after dosing, hens were provided with visual access to a transparent container filled with approximately 20–30 live mealworms. Hens were provided with many mealworms as they had been severely beak trimmed and had trouble picking individual mealworms; providing many mealworms enabled hens to utilise a 'scooping' feeding strategy to consume mealworms if required. The closed food container was positioned at the front of their cage in place of the feeding tray. Mealworm containers had green tape to provide a visual cue to hens. Hens could see the mealworms and could reach the container but could not access them for one minute due to the closed lid. After one minute, the lid was opened, and hens were provided with access to the mealworms for three minutes. Behavioural responses to access to the mealworm container were recorded via GoPro cameras for later analysis. Behavioural analysis was only performed on the fourth (last) day of treatment. Latency to peck the container when the lid was closed and once the lid was removed was calculated as an indicator of motivation to access the mealworms.

### 2.2.4 Foraging tray reward

Exactly 30 minutes after dosing, hens were provided with visual access to a foraging tray half filled with peat moss in the adjacent cage. The adjacent cage was identical to their home cage, with three nipple drinkers and the same space allowance, however, without a feed tray or feed (Figure 2). After three minutes, hens were provided access to the area of the cage with the foraging tray for 10 minutes.

Behavioural responses to access to the foraging tray were recorded via GoPro cameras for later analysis. Behavioural analysis was only performed on the fourth (last) day of treatment. Latency to enter and time spent in the cage area with the foraging tray were calculated and the time spent foraging, dustbathing or pecking at/in the foraging tray was analysed.

**Figure 2 Cage design for positive affective state trial with access to foraging tray**

Taylor 2019.

### 2.2.5 Blood sample collection

Blood samples (4 ml) were collected from all hens from the brachial vein with a 23-guage needle. Samples were collected into syringe and immediately transferred into an EDTA Vacutainer (BD Vacutainer, USA) and mixed 180 degrees slowly 10 times, then stored on ice (4°C) for later processing at the laboratory, but were left for a minimum of 1 hour, but no longer than 4 hours. Samples were centrifuged at 1300G for 10 minutes at 4°C and supernatant was collected. Further centrifugation at 3000G for 10 minutes at 4°C ensured that all platelets were removed. Samples were stored at -20°C and transported on dry ice to Deakin University for analysis.

### 2.2.6 Statistical analysis

The latency to interact, and the proportion of hens interacting, with the foraging tray were analysed with a Kaplan-Meier survival analysis due to the censored nature of the data. Comparisons between treatment groups in time spent in foraging tray, time dustbathing, foraging or pecking were analysed.

## 2.3 Extraction of miRNA

### 2.3.1 Extraction from plasma

Frozen plasma samples were thawed and centrifuged for 3 minutes at 11,000g to remove debris. 300 uL plasma supernatant was used for extraction. The NucleoSpin miRNA Plasma Small RNA isolation kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany) was used, and standard kit protocol was followed for miRNA isolation, with some additions. The optional DNA Digest (50 uL of rDNase) was included. An extra 2-minute centrifuge at 11,000g was included after the last wash step. These additional steps improved miRNA purity and yield as assayed by the Qubit® MicroRNA Assay Kit

(Invitrogen by Thermo Fisher Scientific Co., USA) on a Qubit® 3.0 Fluorometer (Invitrogen by Thermo Fisher Scientific Co., USA) using software APP v1.02 + MCU v0.21.

### 2.3.2 Extraction from egg yolk

The NucleoSpin miRNA Plasma Small RNA isolation kit (Macherey-Nagel, GmbH & Co. KG, Germany) was used for yolk extractions. Standard kit protocol was modified due to high yolk density, which resulted in incomplete homogenisation. As a consequence, increased buffer volumes were required as outlined. Thawed yolks were weighed to obtain a starting sample weight of 0.2 g (equivalent to 200 uL). Lysis Buffer (MLP) volume was increased from 90 uL to 200 uL. Lysis incubation time was increased from 3 minutes to 15 minutes, and included frequent vortexing to improve homogenisation. Protein Precipitation Buffer (MPP) volume was increased from 30 uL to 120 uL. 1 ul glycogen was added to the supernatant during isopropanol addition, as recommended by the kit manufacturers in samples with low yields. Due to additional reagent volume, multiple decants into the spin column were required.

## 2.4 RT-qPCR

Previous genomic sequencing of plasma from chronically stressed hens provided miRNA biomarkers indicative of negative affective state. Five miRNAs (miRNA 2188, miR142-3P, miRNA30c, miRNA10a, miRNA215) were chosen for our diagnostic assay. The assay used RT-qPCR methodology for comparisons between different hen populations (healthy vs stressed). An miRNA commonly found in biofluids, miRUnknown, was included as an endogenous positive control. Prior to RT-qPCR analysis, control (healthy) and treated (stressed) master pools were constructed from the plasma samples. Pooling was recommended by Wade et al. (2016) who used pooling to improve reproducibility and reduce impacts from individual samples with profoundly different miRNA profiles. Each pool was produced from 10 individual samples. The volume required of each sample to contribute 10 ng of miRNA was calculated. This prevented over-representation from individual samples with higher miRNA concentrations in the master pools.

TaqMan® Advanced miRNA Assays (Applied Biosystems by Thermo Fisher, USA) were used in RT-qPCR to quantitate the 5 target miRNAs in the plasma pools. The names of the assays used are listed below (Table 2).

**Table 2 TaqMan miRNA assays incorporated in RT-qPCR analysis**

| miRNA (manufacturer's probe name) | miRNA (chicken annotation) |
|---|---|
| hsa-mir-10a | gga-mir-10a-5p |
| hsa-mir-30c | gga-mir-30c-5p |
| mmu-mir-142-3p | gga-mir-142-3p |
| hsa-mir-215 | gga-mir-215-5p |
| gga-mir-2188 | gga-miR-2188-5p |
| gga-mir-Unknown (Control miRNA) | 5'–CCGAGGCGCCUCGGUGGGC–3' |
| hsa-mir-92a | gga-mir-92-3p |
| hsa-mir-99a | gga-mir-99a-5p |

10 ng of master pool miRNA was used to produce 15 uL cDNA using the Applied Biosystems® TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, USA) as per the manufacturer's instructions, incorporating primers supplied with the probes listed in (Table 2).

Reverse transcription cDNA synthesis was conducted on a Bio-Rad C1000 thermal cycler (Bio-Rad Laboratories Inc, USA) with the following protocol: 30 minutes at 16°C, 30 minutes at 42°C, and 5 minutes at 85°C.

RT-qPCR reactions were conducted in 96-well plates and contained 2 uL cDNA per 20 uL reaction using Applied Biosystems™ TaqMan™ Fast Advanced Master Mix (18 uL) (Applied Biosystems by Thermo Fisher Scientific, USA). A maximum of 6 miRNAs were assayed per cDNA reaction. Assays were run in duplicate alongside no template controls (NTC) for each target miRNA to detect reagent contamination. Assays always contained a mix of stressed and healthy samples to account for inter-run variations or bias on the analyser. RT-qPCR was conducted on a Bio-Rad CFX96 real-time system (Bio-Rad Laboratories Inc, USA) using the following protocol: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, with fluorescence read at the end of each 60°C step. Data was retrieved from the Bio-Rad CFX manager software version 3.0.1215.0601 (Bio-Rad Laboratories Inc, USA). Cycle Threshold (Ct) values were recorded for each miRNA. An miRNA was classified as amplified or detected if the Ct value was less than 35. Markers amplifying after Ct 35 show low precision as depicted by high standard deviations. For the plasma samples, the quality criteria implemented for a successful run was a Ct for miR2188 less than 25, no miRNA Cts greater than 35 and standard deviations between Cts for each miRNA less than 0.5. Amplification of the miRUnknown control within the stipulated quality control criteria was used as an internal indicator of successful cDNA synthesis.

RT-qPCR was conducted on miRNAs extracted from egg yolks from control (healthy) and treated (stressed) hens. TaqMan® Advanced miRNA Assays (Applied Biosystems by Thermo Fisher, USA) were used. We sought to replicate previous work by Wade et al. (2016), the first to isolate miRNAs from egg yolk. Of the 8 miRNAs they originally isolated, we successfully detected 3 (miRNA30c, miRNA92a, miRNA99a).

## 2.5 Statistical analysis

Average expression (Ct values) of the 5 target miRNAs in the 3 control master pools (C1, C2, C3) formed the healthy average miRNA profile. All pool samples were run 3 times with average Ct values for each miRNA calculated. For negative affective state trial samples, miRNAs were allocated markers A, B, C, D or E based on order of amplification (A earliest, E latest) as outlined in (Figure 3).

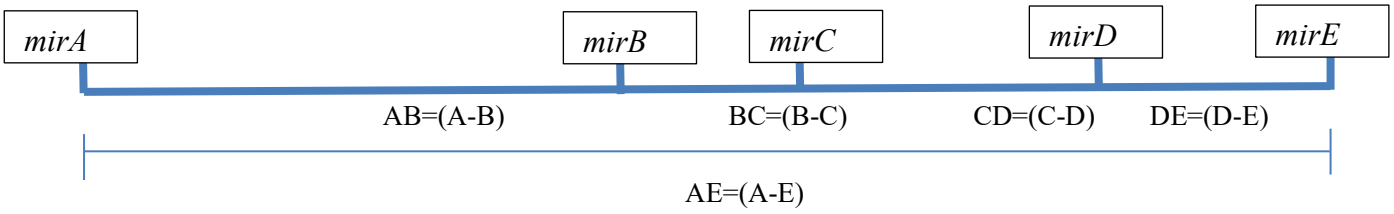


**Figure 3 miRNAs assigned marker labels based on Ct value determined by RT-qPCR**

First miRNA to amplify is assigned marker A (highest expression).
Last miRNA to amplify is assigned marker E (lowest expression).

The average control Ct values depict the expected miRNA profile in a healthy hen population. Pooling helps account for biological variability in the normal healthy population. Using this background for comparison, blind samples were analysed against the same 5 target miRNAs to determine their origin as stressed or healthy. Healthy samples were expected to exhibit similar miRNA expression to the controls whilst stressed samples would show divergence. Relative expression of the 5 target miRNAs was used to calculate a single number known as a Difference Score. The Difference Score determines if a sample originated from a stressed or healthy hen (Wade et al. 2018).

## 2.6 Sequencing

Extracted plasma miRNA from 80 positive affective state trial samples (n = 20 foraging control, n = 20 foraging Nalmefene, n = 20 mealworm control, n = 20 mealworm Nalmefene) and 40 negative affective state trial egg yolks (n = 5 control day 5, n = 5 stress day 5, n = 15 control day 9, n = 15 stress day 9) were sent to Qiagen Genomic Services Department (Qiagen Inc, Hilden, Germany) for genomic sequencing. The positive affective state plasma samples were sequenced to detect differential miRNA expression between the control (reward pathway activated) and the treated (reward pathway blocked). The negative affective state yolk samples were sequenced to ascertain if a stress profile similar to that detected in plasma is transferred in the egg.

# 3 Results and discussion

## 3.1 Negative affective state trial

The negative affective state trial centred on comparison between hens exposed to chronic stress and healthy hens reared in the same production system. This was an important stipulation for the trial, since Wade et al. (2018) demonstrated how hens of different ages and reared in different production systems express differing basal miRNA profiles (free range > barn > cage). It was important to ensure miRNA variations were resulting from stress exposure rather than as a by-product of the hen's production systems. To achieve this and reduce the risk of spurious results, we compared hens of the same age from the same production system. Induction of social stress occurred when cage-mates were changed every day and birds commenced fighting for resources. Stress induction was validated through the tonic immobility (TI) behavioural test. This test indicated that stressed hens were more fearful than control hens as indicated by a longer duration spent in TI (7.5% of control hens versus 15.8% treatment hens reached maximum of TI).

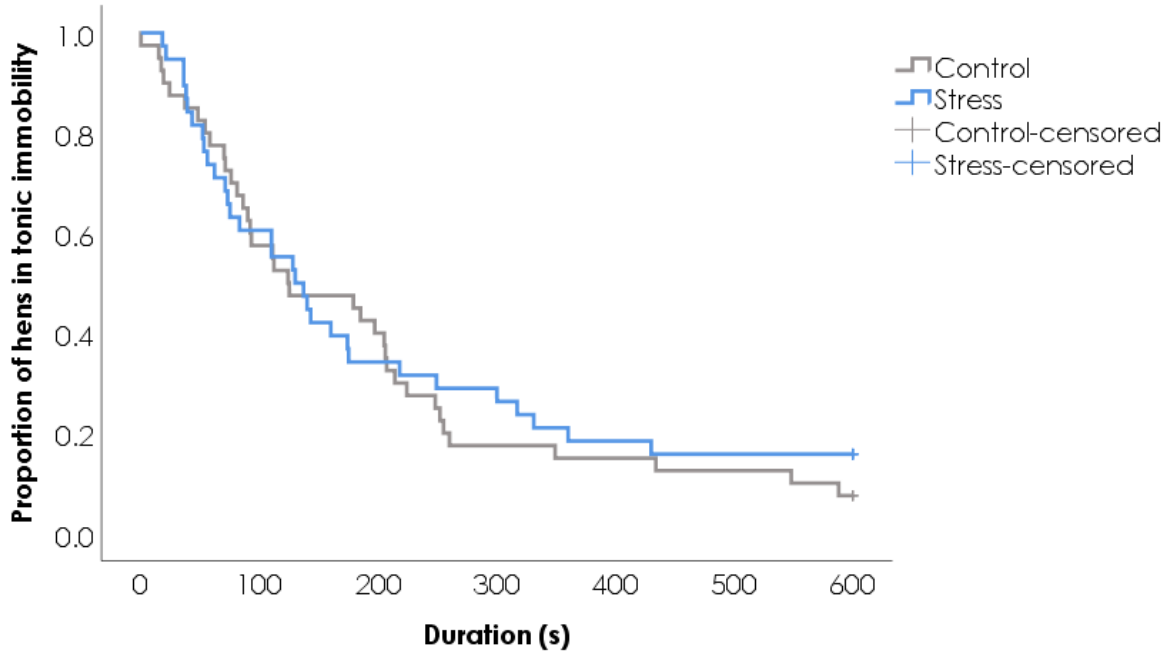


**Figure 4 Kaplan-Meier curves indicating the proportion of control (grey line) and treatment (blue line) hens that came out of the tonic immobility (TI) state (y-axis) over time (seconds) (x-axis) after 10 continuous days of mixing (treatment) or no mixing (control)**

Every time a hen came out of the TI state the probability on the y-axis drops.

### 3.1.1 Plasma

The average miRNA concentration extraction from plasma was 9.87 ng/uL. Results of the RT-qPCR runs were used to calculate the average Ct values for the target miRNAs in control (healthy) and stressed pools. A consistent miRNA pattern was identified, as shown in Figure 5.

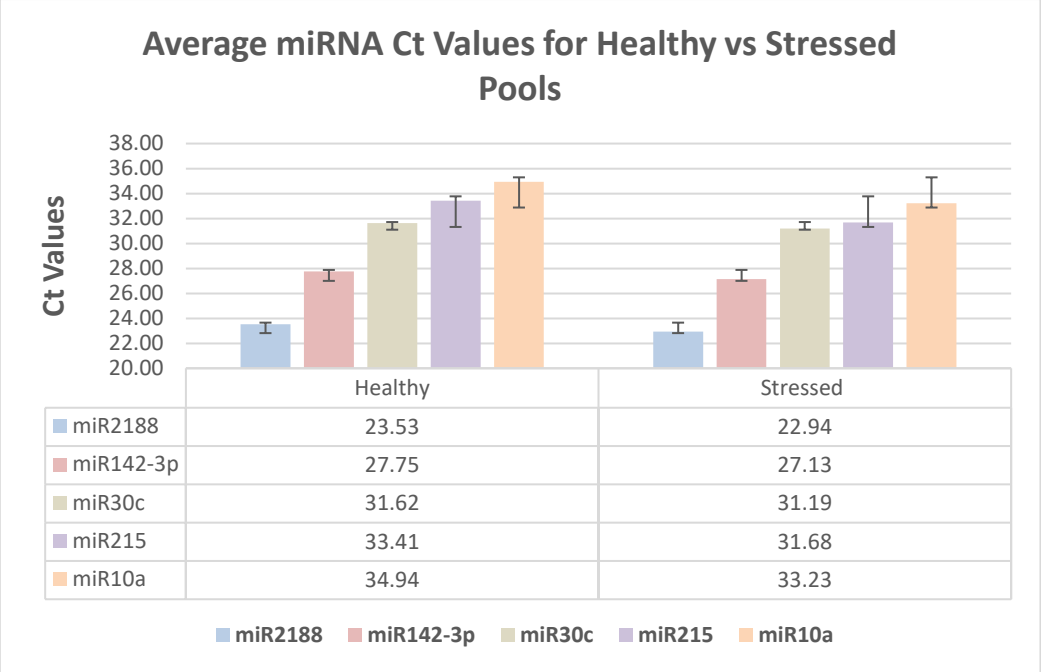


**Figure 5 Average Ct values for target miRNAs derived from RT-qPCR analysis**

Note minimal alteration in absolute Ct values between healthy and stressed populations.

An miRNA was deemed detected if it amplified prior to the 35$^{th}$ cycle of RT-qPCR. miRNAs amplifying after Ct 35 show low precision, as demonstrated by high standard deviations between Ct values. Any run showing amplification in the no template control (NTC) wells was excluded due to possible reagent contamination. Samples were initially assayed as singular specimens to demonstrate miRNA quality and reproducibility. Suitable candidates (10 individual samples per pool) were randomly selected to construct master pools (control and stressed). The Ct values for each target miRNA in the 3 control master pools (C1, C2, C3) were converted to log-ratios in Excel (Figure 6) and from these values the Healthy Difference Score was derived.

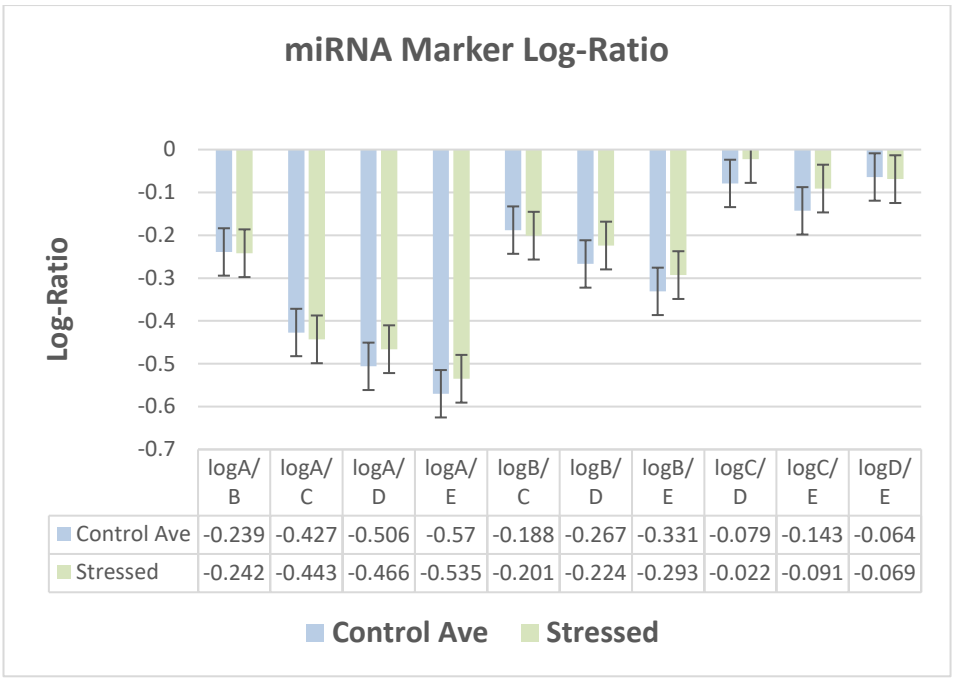


**Figure 6 Log-ratio of miRNA markers labelled A-E based on amplification order (A first, E last) Ct values**

Log-ratios magnify small alterations between miRNA expression. This methodology was chosen due to its robustness in detecting the relative miRNA differences between a normal healthy hen population and a stressed population. The tool used to capture this divergence is the Difference Score, the sum of sample miRNA log-ratios minus the healthy log-ratios derived from the 3 control pools. Using the Difference Score (DS) methodology, we were able to successfully construct an assay capable of ascertaining whether blind samples originated from stressed or healthy layer populations. As such, the assay successfully predicted that samples with a high DS originated from hens experiencing a negative affective state due to stress exposure. Examples of DS generated from stressed and healthy hen populations are given in (Table 3).

**Table 3 Difference score calculations based on average healthy log-ratios calculated from 3 control pools (C1, C2, C3)**

| | C1 | C2 | C3 | Healthy Ave | Stressed Pool | Healthy Pool |
|---|---|---|---|---|---|---|
| logA/B | -0.2763 | -0.2072 | -0.2333 | -0.2389 | -0.0031 | -0.0374 |
| logA/C | -0.4410 | -0.4216 | -0.4180 | -0.4268 | -0.0164 | -0.0141 |
| logA/D | -0.5151 | -0.5026 | -0.5005 | -0.5061 | 0.0404 | -0.0090 |
| logA/E | -0.5705 | -0.5622 | -0.5777 | -0.5701 | 0.0355 | -0.0003 |
| logB/C | -0.1647 | -0.2144 | -0.1847 | -0.1879 | -0.0133 | 0.0232 |
| logB/D | -0.2388 | -0.2954 | -0.2672 | -0.2671 | 0.0435 | 0.0283 |
| logB/E | -0.2942 | -0.3550 | -0.3444 | -0.3312 | 0.0386 | 0.0370 |
| logC/D | -0.0741 | -0.0811 | -0.0825 | -0.0792 | 0.0568 | 0.0051 |
| logC/E | -0.1295 | -0.1406 | -0.1598 | -0.1433 | 0.0519 | 0.0138 |
| logD/E | -0.0553 | -0.0596 | -0.0773 | -0.0641 | -0.0049 | 0.0087 |
| | | | | **Difference Score** | **0.2289** | **0.0554** |

Note higher DS in stressed sample compared to healthy sample.

### 3.1.2 Eggs

The average yolk miRNA concentration was 14.72 ng/uL. Validation of yolk miRNAs was conducted via RT-qPCR using target miRNAs as demonstrated in Figure 7 and previously isolated from egg yolk by Wade et al. (2016). miRNAs that appeared reliably prior to cycle 35 were deemed valid and consequently four of the eight miRNAs tested passed (Figure 7).

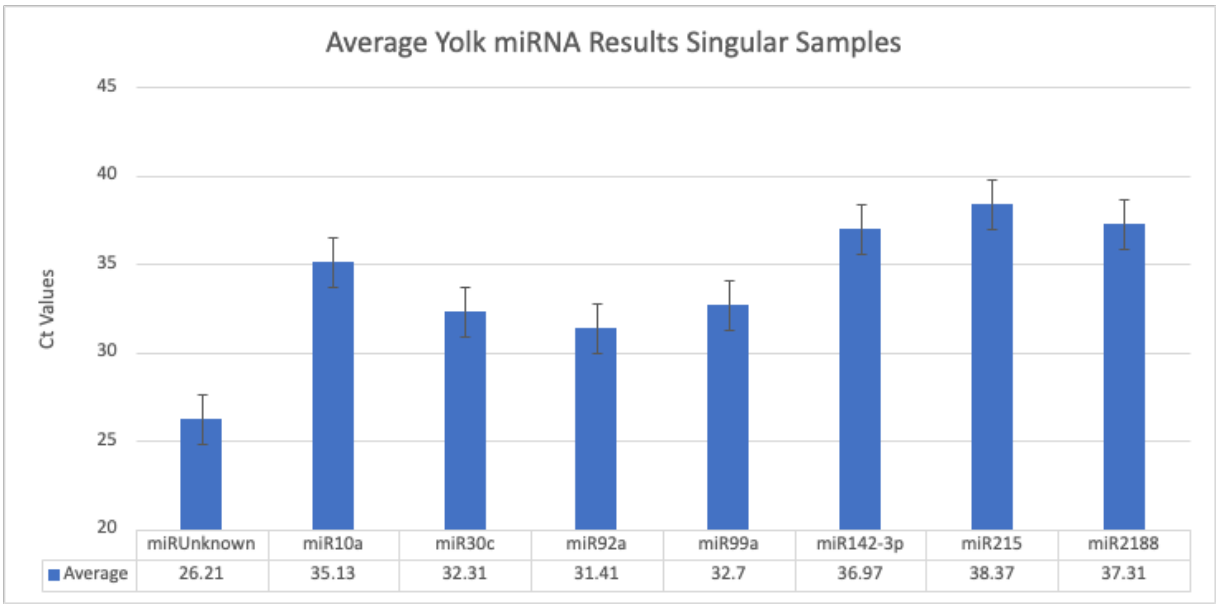


**Figure 7 Average yolk miRNA RT-PCR results for each of the previously identified miRNA stress biomarkers**

Further to detecting the miRNAs in singular samples, pooled samples were also tested. Pooled samples are of the utmost importance as it is unrealistic to sample hens on an individual basis within industry, rather they need to be sampled on a flock basis, and the best way to achieve this is using pooled samples. Pooled samples were created from day 5 and day 9 eggs combined; each pool (Control and Treated/Stressed) was constructed from 8 samples (4 day 5 and 4 day 9 samples). We chose two time points to further ensure that our assay would be more robust in the field and to avoid the assay detecting a single point in time rather that the underlying stress profile of the birds. Figure 8 demonstrates that there were indeed differential expression patterns detected in the pooled samples for each of the four miRNAs tested. This result demonstrates that we have successfully demonstrated the validity of a non-invasive test for welfare in hens.

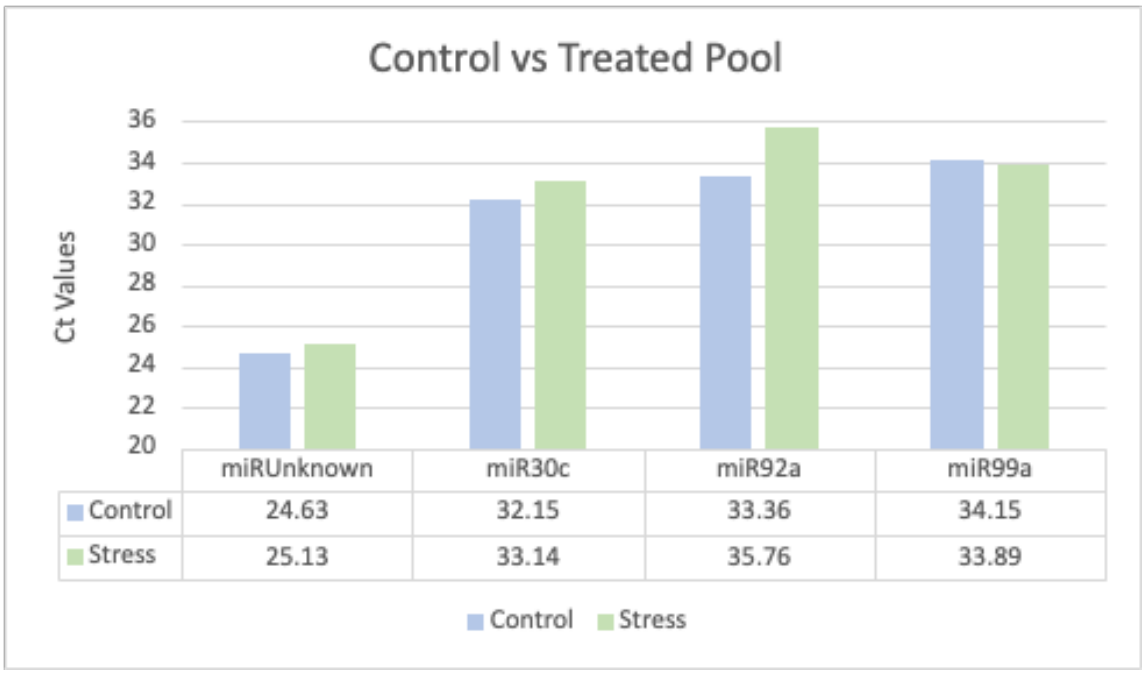


**Figure 8 Comparison of pooled samples (days 5 and 9) from both control and stress induced samples using RT-PCR**

## 3.2 Positive affective state trial

Induction of a positive affective state was validated by more control hens engaging with both food (closed and opened mealworm containers) and non-food (accessing the foraging tray) rewards than the Nalmefene-treated hens. This indicates that Nalmefene administration successfully inhibited the reward pathway as evidenced by reduced anticipatory behaviours displayed by the treated hens.

More control hens pecked the closed mealworm container and were quicker to do so than hens from the Nalmefene treatment group ($\chi 2$ (1, 50) = 8.32, p = 0.004; Figure 9). More control hens pecked the open mealworm container and were quicker to do so than hens from the Nalmefene treatment group ($\chi 2$ (1, 50) = 5.36, p = 0.021; Figure 10).

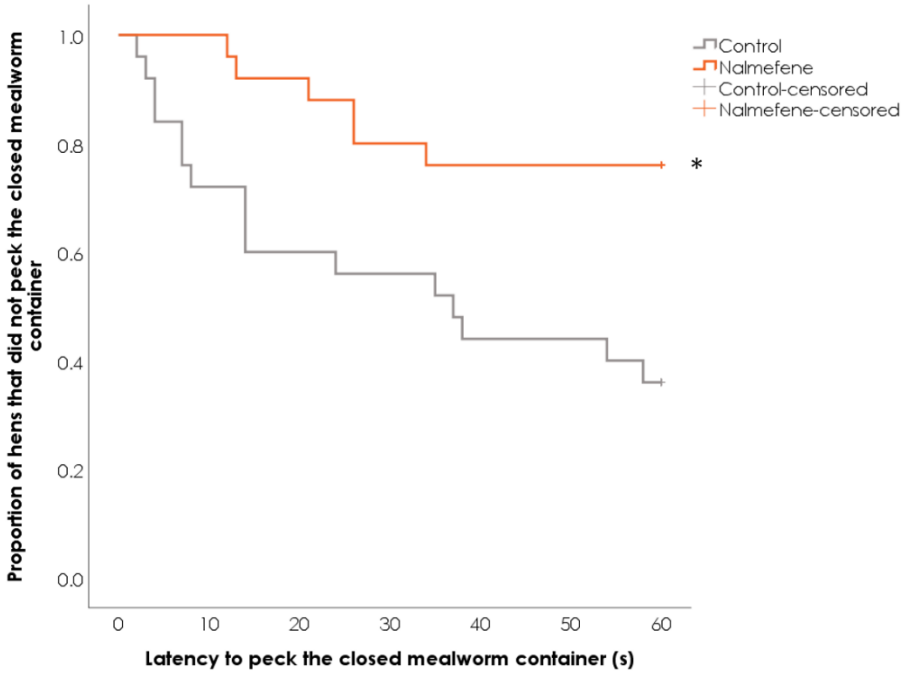


**Figure 9 Kaplan-Meier curves indicating the proportion of control (grey line) and treatment (orange line) hens that pecked the closed mealworm container (y-axis) over time (seconds) (x-axis) on the fourth day of testing**

Every time a hen pecked the closed container the probability on the y-axis drops.
The closed mealworm container was provided to hens for 60 seconds 30 minutes after dosing with either saline (control) or Nalmefene (treatment).
* indicates a significant difference between treatment groups at $p < 0.05$.

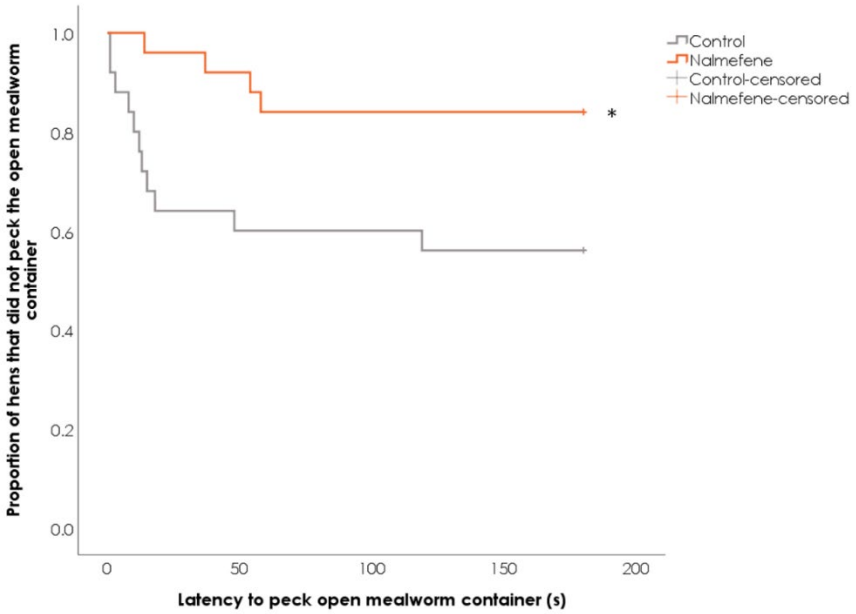


**Figure 10 Kaplan-Meier curves indicating the proportion of control (grey line) and treatment (orange line) hens that pecked the open mealworm container (y-axis) over time (seconds) (x-axis) on the fourth day of testing**

Every time a hen pecked the open container the probability on the y-axis drops.
The open mealworm container was provided to hens for 3 minutes 30 minutes after dosing with either saline (control) or Nalmefene (treatment).
* indicates a significant difference between treatment groups at $p < 0.05$.

### 3.2.1 Non-food reward

More control hens entered side B of the cage (foraging tray side), and were quicker to do so when permitted access than control hens after four consecutive days of exposure to the new cage and foraging tray ($\chi 2$ (1,48) = 5.07, p = 0.024; Figure 11).

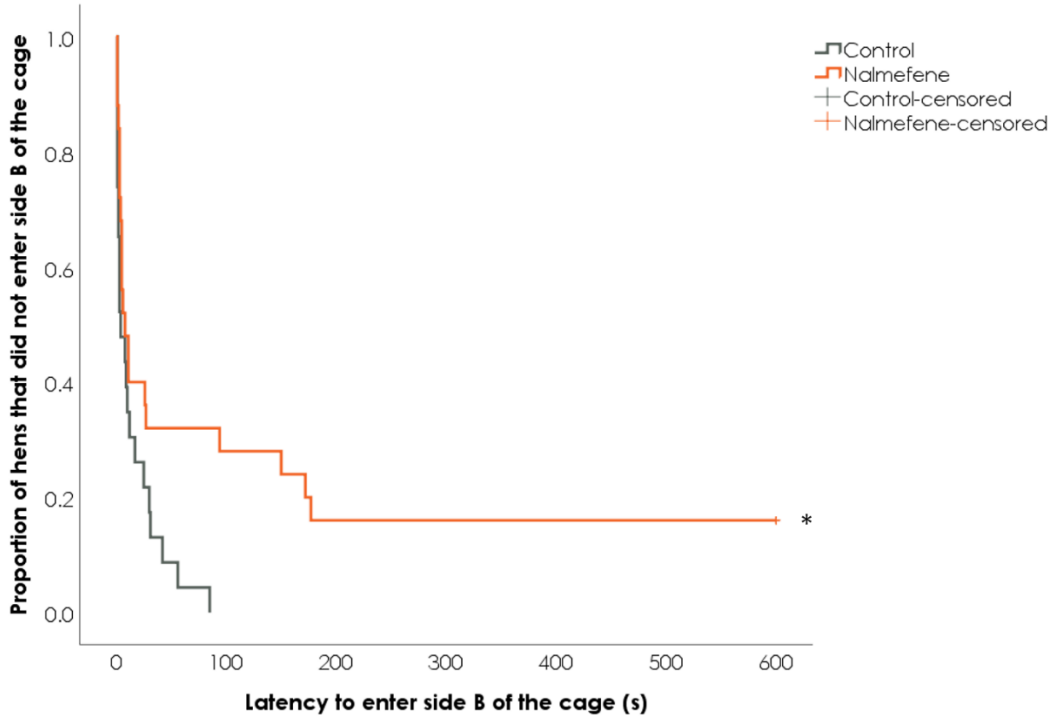


**Figure 11 Kaplan-Meier curves indicating the proportion of control (grey line) and treatment (orange line) hens that entered side B of the cage (y-axis) over time (seconds) (x-axis) on the fourth day of testing**

Every time a hen entered side B of the cage the probability on the y-axis drops.
Side B of the cage was only available for ten minutes each day of testing 30 minutes after dosing with either saline (control) or Nalmefene (treatment).
Side B contained a foraging tray containing peat moss.
* indicates significant difference between treatment groups at $p < 0.05$.

More control hens interacted with the foraging tray, and were quicker to do so, than the Nalmefene treated hens ($\chi 2$ (1, 48) = 6.89, p = 0.009; Figure 12). Both control and Nalmefene treatment hens spent most of the time pecking when interacting with the foraging tray. Control hens spent more time interacting with the foraging tray than treatment hens (control 60.1 ± 7.4%; treatment = 31.9 ± 6.8%; U = 151.5, p = 0.005; Figure 13). Specifically, Nalmefene hens spent more time dustbathing and pecking in the foraging tray than control hens (dustbathing – control 13.8 ± 5.5%; treatment 5.11 ± 3.7; U = 205.5, p = 0.044; pecking – control 33.2 ± 6.3; treatment – 16.6 ± 3.6; U = 189.5, p = 0.043; Figure 13). There was no difference in the time spent foraging between control and treatment hens (control 12.8 ± 4.4%; treatment 10.2 ± 4.3%; p = 0.172; Figure 13).

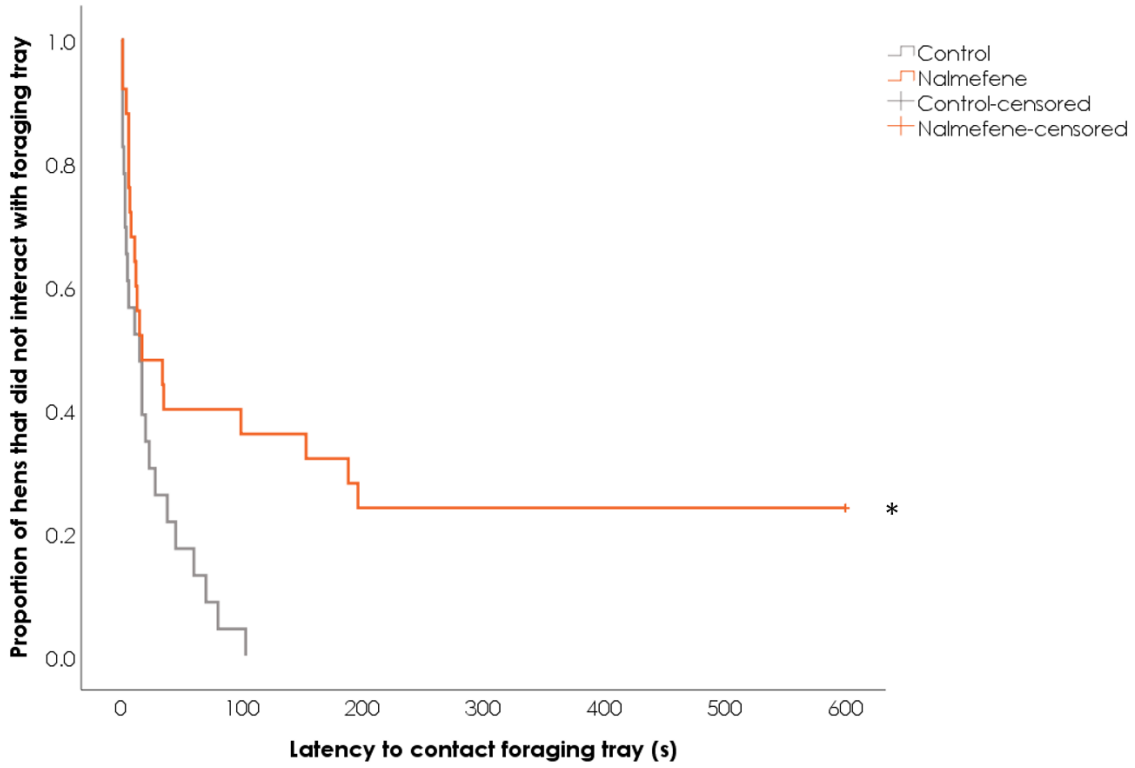


**Figure 12 Kaplan-Meier curves indicating the proportion of control (grey line) and treatment (orange line) hens that interacted with the foraging tray (y-axis) over time (seconds) (x-axis) on the fourth day of testing**

Every time a hen pecked the container the probability on the y-axis drops.
* indicates significant difference between treatment groups at $p < 0.05$.

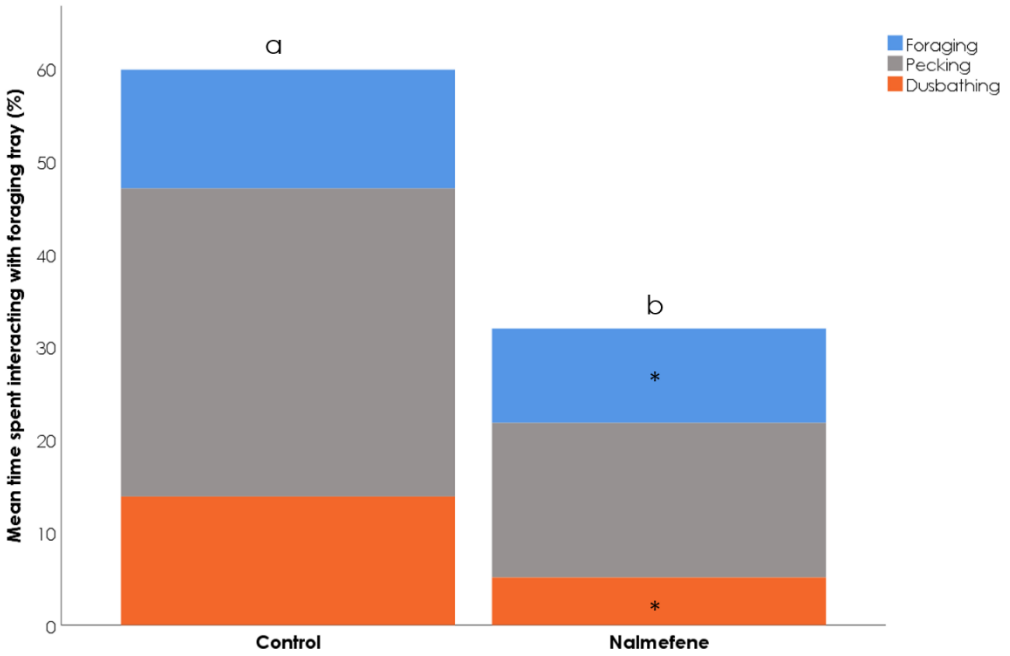


**Figure 13 Mean time spent interacting with the foraging tray (%) for control and Nalmefene treatment hens**

Stacked colours within bars indicate the time spent performing specific behaviours when interacting with the foraging tray – dustbathing (blue), foraging (grey) or pecking (orange).
Differing subscript indicates a significant difference of time spent interacting with the foraging tray between treatment groups at $p < 0.05$.
* indicates a significant difference of time spent performing a specific behaviour in the foraging tray between treatment groups.

We were able to successfully induce a state of reward in hens by the provision of both food and non-food rewards. Additionally, we were able to block the reward state in hens with the administration of Nalmefene, evident by less interaction with rewards (mealworm or foraging tray) and a longer latency to access rewards when they were accessed by Nalmefene hens, relative to control hens that did not receive the drug.

Of note, relative to a previous pilot trial that aimed to validate the Nalmefene treatment, fewer hens pecked the closed or open mealworm container. This may be related to a shorter period of time that hens were exposed to the mealworm container (both closed and open) or the severe beak trimming that hens had undergone before arriving at the UNE poultry research facility.

### 3.2.2 Plasma

The average plasma miRNA concentration was 4.28 ng/uL. Sequencing was used to isolate miRNA profiles and detect differential expression between sample groups (control and treated). Control groups for foraging and mealworm were expected to exhibit an miRNA profile indicative of a positive affective state. This profile should not be present in the Nalmefene-treated groups. Sequencing detected the miRNAs listed in Table 4 as differentially expressed between treatment groups.

**Table 4 miRNAs identified by genomic sequencing present in positive award state (control, C) hens compared to Nalmefene-treated (no positive affective state, N) hens**

| F/C vs F/N | F/C vs M/C | M/C vs M/N | F/N vs M/N |
|---|---|---|---|
| gga-miR-3528 | gga-miR-29a-3p | **None** | gga-miR-140-3p |
| gga-miR-122-5p | gga-miR-29c-3p | | gga-miR-122-5p |
| gga-miR-338-3p | gga-miR-17-5p | | gga-miR-206 |
| | gga-miR-9-5p | | gga-miR-16c-5p |
| | gga-miR-181a-3p | | gga-miR-3528 |
| | gga-miR-142-5p | | gga-miR-12211-5p |
| | | | gga-miR-9-5p |
| | | | gga-miR-1a-3p |
| | | | gga-miR-1b-3p |
| | | | gga-miR-29a-3p |
| | | | gga-miR-181a-3p |
| | | | gga-miR-15b-5p |
| | | | gga-miR-29c-3p |
| | | | gga-miR-17-5p |
| | | | gga-miR-140-5p |
| | | | gga-miR-126-5p |
| | | | gga-miR-144-3p |
| | | | gga-miR-30a-5p |
| | | | gga-miR-219b |
| | | | gga-miR-1559-5p |
| | | | gga-miR-1451-5p |
| | | | gga-miR-12288-5p |
| | | | gga-miR-32-5p |
| | | | gga-miR-103-3p |
| | | | gga-miR-101-3p |
| | | | gga-miR-181b-1-3p |
| | | | gga-miR-143-5p |
| | | | gga-miR-20b-5p |
| | | | gga-miR-15a |
| | | | gga-miR-456-3p |
| | | | gga-miR-499-5p |
| | | | gga-let-7b |
| | | | gga-miR-181a-5p |
| | | | gga-miR-3538 |
| | | | gga-miR-144-5p |
| | | | gga-let-7i |
| | | | gga-miR-193a-5p |
| | | | gga-miR-30e-3p |
| | | | gga-miR-29b-3p |
| | | | gga-miR-142-5p |
| | | | gga-miR-126-3p |
| | | | gga-miR-6651-5p |
| | | | gga-miR-1662 |
| | | | gga-miR-2188-3p |
| | | | gga-miR-1682 |
| | | | gga-miR-1434 |
| | | | gga-miR-3523 |
| | | | gga-miR-130b-3p |
| | | | gga-miR-182-5p |
| | | | gga-let-7g-5p |
| | | | gga-miR-125b-5p |
| | | | gga-miR-425-3p |
| | | | gga-miR-155 |

FC=foraging control, FN=foraging Nalmefene, MC=mealworm control, MN=mealworm Nalmefene.

The large difference in profiles between the two Nalmefene groups is unusual and was not expected. We expected these groups to show the most similarity in miRNA expression between the 4 groups. Differences between the control groups may be explained by the cognitive pathways being activated in the different activities, foraging and mealworm consumption. The 2 mealworm groups (M/C and M/N) showed no differential expression. This may be the result of ineffective Nalmefene administration. Sequencing of the trial yolks successfully identified 18 of the original 21 yolk miRNAs tested by Wade et al. (2016). We were able to replicate isolation of these miRNAs in all samples and provide evidence that it will be possible to develop this further into a biomarker test, following more trials and testing of industry samples.

# 4 Strength and limitations

Hens of different ages and from different production systems (cage, barn, free range) show variation in miRNA profiles. This impacts log-ratio values and DS calculation. We are cognisant of the fact that testing samples from mixed aged hens and mixed production systems using the current methodology may produce erroneous results. Hence, a much wider pool of samples will need to be tested to ensure that we can account for these fluctuations.

Currently, for this methodology to be successful, samples for control master pool construction must be identified by their population origin (stressed or healthy). Controls will change with sample batches and require testing and quality controlling for each batch. To date there is no universal control miRNA profile that encapsulates all healthy samples. The ideal would be to have an assay that is capable of testing all samples blindly. Further work involving more in-depth industry sampling would be required to enable this.

During optimisation of the extraction protocol, commercial or domestic eggs were trialled with liquid yolks able to be pipetted. When the project eggs arrived, the yolks were solid and unable to be pipetted, so an equivalent weight of yolk was weighed out for extraction (0.2 g yolk in place of 200 uL yolk). As well as being problematic to extract, the yolk samples also showed reverse transcription inhibition when tested by the sequencing laboratory. This problem was solved by reducing the input volume for cDNA synthesis, which reduced inhibitors and allowed for library preparation and sequencing.

# 5 Future directions

To date, the DS methodology for miRNA stress detection is a research tool only. It has the potential to be refined and developed into a point of testing industry tool by broadening the sampling pool, which will facilitate deeper genomic sequencing. It will be important to test industry samples from a broad range of housing systems to ensure that the test and methodology will stand up in industry. It is likely that more miRNAs will be added to the biomarker profile during this process. This will assist in strengthening knowledge of the miRNA stress profile. As we have demonstrated that the biomarker profile can be detected in eggs, we will be able to pursue the non-invasive testing in eggs for industry samples in the future.

Likewise, further testing would need to be performed prior to the positive affective state being used as an industry tool. This project has developed the tools required to investigate positive affective states and hence further research would only need to look at strengthening the biomarker profile. Once established, a positive affective state biomarker test would be able to be used to assess different enrichments used in industry and also assess new or changed housing systems. This test, like the stress test, could be performed non-invasively in eggs further highlighting the potential for use in industry whole flock applications.

Further research could be conducted to establish the exact timeline of when miRNAs are packaged into eggs. Limited knowledge exists about the epigenetic impacts of stress miRNA on the developing chick. It would be of value to further interrogate the longevity of the different affective states and if are they reversible. Further work defining the timeline of miRNA transmission into eggs and the potential epigenetic influences of transmitted miRNAs on the developing chick would be recommended. These studies would provide valuable insights and enable interventions to be made in the future, especially in the early stages of chick growth.

# 6 Conclusions

This research has successfully met both aims: (1) the development of a non-invasive stress biomarker test; and (2) the ability to measure different affective states in laying hens. This research has provided the industry with evidence-based, non-invasive tools for assessing and measuring hen welfare. While the tests are still in their infancy, we have demonstrated that they indeed work and will have future applicability in industry. We have confirmed that it is possible to use eggs, specifically yolks to determine the stress levels in the hens. Further we have developed this test to work in pooled samples using RT-PCR, providing a more viable and cost-effective test for industry as opposed to individual sequenced samples. While we acknowledge that this test is not industry ready just yet, we are confident that we have laid the foundations and that with further industry testing we will have delivered a viable and effective stress biomarker test. This research has for the first time demonstrated that we can illicit and test positive affective states in hens. This ground breaking research has developed and validated the tools required to test positive affective state in hens and has identified a preliminary miRNA profile in plasma.

These tools have the potential to enable the broader community to obtain an understanding of the current affective state of welfare of laying hens across all commercially relevant production systems. The Australian poultry industry, namely chicken egg producers, are the main beneficiary of these project outcomes. These evidence-based tests, if adopted by the industry, would provide a means for accurately determining good and poor welfare status within a flock. This is crucial to the industry as producers are continually questioned and tested with regard to the status of welfare in their production systems.

Currently. many of the large egg buyers (supermarkets) are setting guidelines on how eggs are to be produced in terms theoretically based on the welfare state of layers but with no means of quantitative measurement. While good welfare of layers is the goal for both producers and consumers, many of the guidelines presently being formulated are based on consumer perceptions rather than facts. The tests developed in this project seek to provide the industry with reliable and simple tests that the industry can use to monitor the welfare status of production systems, and which will enable it to make informed decisions about management changes. This project provides tools that will have a significant impact in social terms, as the industry will be able to directly report on the welfare status of all poultry egg production systems.

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