

Personality and sex: stress physiology effects
on vocal performance in the common myna
(Acridotheres tristis)

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Abstract

Physiological stress responses are essential to vertebrate survival. However, the strength of stress responses differs among individuals, even within the same species, population and sex. Differential stress reactivity can provide selective advantages depending on ecological context. In songbirds, individual quality can be honestly reflected through song quality, as factors like long-term stress and reduced energy reserves can reduce individual song quality. Variation in song is well-studied in males and likely shows similar patterns in females, but female song is poorly researched. My research examines how individual stress reactivities and sex interact with short-term stress to alter song complexity in adult songbirds.

I investigated these relationships using wild-caught common mynas (*Acridotheres tristis*), scoring individual stress reactivity, measuring physiological stress and recording vocalisations at baseline stress. Then the birds were fed corticosterone (CORT) to simulate a short-term stress response, and I measured changes in stress, behaviour and vocalisations. Stress was measured using faecal CORT and eye temperature with a thermal imaging camera, a new non-invasive stress measurement method. To quantify stress reactivity, I conducted three neophobia tests and rated behavioural stress during handling. I created a stress reactivity score using a principal component analysis of those measures. Eye temperature significantly increased after CORT ingestion, measured through a thermal camera, and was correlated with faecal CORT concentrations, indicating it is an effective estimate of stress response.

Simulated short-term stress significantly increased singing activity and song complexity, potentially indicating an adaptive response to CORT activation. Individuals that were more stress reactive (behaviour and handling) had smaller physiological responses to CORT treatment but more complex songs and higher singing activity. Males had higher song complexity during baseline conditions, but female songs became similarly complex when CORT was elevated. The effects of stress reactivity on singing were sex-specific in many parameters, raising many interesting questions for future research.

My research findings illustrate that individual differences in responses to stressors, such as ecological disturbances, can have substantial consequences for other, seemingly unrelated but ecologically relevant traits (singing) and also identifies new avenues for research on sex-specific relationships between stress and song. These findings are essential as stress-inducing disturbances increase in frequency under a changing climate.

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1. Introduction

Physiological stress responses are essential to vertebrate survival (Lu et al. 2021). Stress responses allow individuals to respond to warnings detected by their nervous systems, such as low blood sugar, pain, and fear (Goldstein and Kopin 2007). Individuals within the same species differ in how they respond to stress, with some individuals being more reactive to stress than others, even to the same stimulus. These individual differences have been documented extensively especially in birds (Cockrem 2007). Interestingly, bird song reveals substantial information about individuals, such as individual quality, age, sex, and even chronic stress (Buchanan et al. 2003, Langmore 1998, Nottebohm 1970, Spencer et al. 2003). However, despite findings that birdsong is affected by many factors, including oxidative stress (Casagrande et al. 2016), the effects of short-term stress on song complexity remains unexplored. Since individual personality can affect direct and indirect fitness (Dingemanse et al. 2004), song complexity may also be affected by an individual's stress reactivity, especially when experiencing short-term stress.

These relationships are essential to investigate since bird song is a fundamental behaviour, having critical roles in communication, survival and reproduction in both sexes for many species (Gil and Gahr 2002). How sex influences these relationships may further inform these questions about song complexity (Odom et al. 2014).

1.1 Quality and song differences

Bird vocalisations play a crucial role in survival and reproduction. They are critical for communication, act in various social contexts across bird taxa, and vary depending on environmental context (Catchpole and Slater 2003, Marler and Slabbekoorn 2004, Williams 2004). Different types of vocalisations often have specific functions. For example, birds use food calls to communicate with conspecifics about food resources, alarm calls convey information about dangers such as predators, and agonistic calls are essential for defending territorial boundaries (Marler and Slabbekoorn 2004, Williams 2004). Songs are a particular class of vocalisation that are usually associated with a display of quality, functioning to attract mates or deter conspecifics from territories for example (Marler 2004). Birdsong is most well-known and well-studied in sexual selection, where males use songs to attract mates, and females prefer better and more complex songs (Ballentine et al. 2004). However,

birdsong is also widespread and used by females, often for female-female competition (Cain et al. 2015, Langmore 1998, Odom et al. 2014).

Singing is a costly behaviour, and as such, it is often used as a proxy for health and individual quality and degrading in quality as health declines (Casagrande et al. 2016). Singing increases metabolism, and more complex songs require higher oxygen consumption (Oberweger and Goller 2001). This increase in metabolic energy through singing produces heat and can be directly measured as a proxy of the cost of each song (Ward and Slater 2005). Consequently, it is energetically costly to produce a complex song, therefore, song quality and singing activity can indicate a bird's physiological status and current energy reserves (Catchpole and Slater 2003, Gil and Gahr 2002). Thus, bird song is considered an honest indicator of quality for both males and females, as healthier individuals tend to sing more complex, longer or louder songs (Cain et al. 2015, Gil and Gahr 2002, Kipper et al. 2006). This dynamic is analogous to how nutritional stress negatively influences ornamental bird plumage (McGraw et al. 2002). As they signal individual quality complex songs can have direct fitness benefits, such as mate attraction, and indirect fitness benefits, such as resource defence (Boogert et al. 2008, Cain et al. 2015, Nemeth et al. 2012, Odom et al. 2021, Woodgate et al. 2012). These benefits are predicted to outweigh the metabolic costs in healthy or high-quality individuals.

In the oscine songbirds, singing is a learned behaviour that requires several periods of learning that are essential for a bird to sing its proper song (Nottebohm 1970). This learning process includes memorising the 'correct' song (learned from a tutor) during the sensory phase (Beecher and Brenowitz 2005) until, eventually, the song is fixed (crystallisation, Nowicki et al. 1998). Therefore, disruptions that impact individual quality during these periods, such as stress, can reduce the quality of song throughout a bird's life. The song learning process varies between species however, as many songbird species finish learning after crystallisation, and some species continue to learn new songs throughout their life (Chaiken and Böhner 2007).

Developmental stressors (e.g. nutritional stress or oxidative stress) often affect fitness later in life (Bell et al. 2018, Noguera et al. 2012). The developmental stress hypothesis proposes that stress during the juvenile song learning stage can have lasting effects on song quality in adult birds (Buchanan et al. 2003, Spencer et al. 2003). This hypothesis initially proposed that these lasting effects result from interference in neural development following stress

experienced prior to song crystallisation (Nowicki et al. 1998). However, further research supports early developmental stress as influential in affecting learning accuracy and copying songs later in life (Buchanan et al. 2003). Experiments have repeatedly supported the developmental stress hypothesis, suggesting that an individual's past stressors negatively affect later life, primarily through song (Spencer et al. 2003, Wada et al. 2007, Yamada and Soma 2016).

Studies examining factors that affect adult song performance have heavily focused on developmental stressors. As a consequence, the effect of short-term stressors remains relatively unknown. However, short-term stress is likely to affect adult birdsong since stressors experienced as an adult can influence individual fitness due to reductions in physiological quality (Partecke et al. 2006). For example, changes in nutrient intake from short-term fasting can induce stress and negatively impact individual quality during adulthood (Lynn et al. 2010). Beyond nutritional deficiencies, likely short-term stressors include predator encounters, harsh to extreme weather conditions, losing a fight with a conspecific, and any other events that may increase circulating corticosterone (CORT), a hormone that increases in stressful situations, see below. The effects of short-term stress on adult birdsong may also present differently according to sexes due to links between the hypothalamic-pituitary-adrenal (HPA) axis and sex steroids, affecting males and females differently (Dickens and Bentley 2014).

Though female song is widespread and often serves similar competitive and signalling roles, the effects of any type of stressor on song in females are entirely unexplored. As song often signals quality, it is usually considered a classically sexually selected trait, well known in males for its function in advertising reproductive quality in the context of female choice and male-male competition, such as occurs in swamp sparrows (*Melospiza georgiana*, Ballentine et al. 2004, Gil and Gahr 2002). However, female song is ubiquitous and is the ancestral state for songbirds, although it has been repeatedly lost in many lineages (Odom et al. 2014). Most cases of female song present evidence for socially driven functions of female song, operating in female-female competition over resources, in gaining and defending territories, where success has direct fitness benefits on nesting success (Brunton et al. 2016, Cain et al. 2015, Cain and Ketterson 2012). These benefits seem to drive the pattern of female song, being more common in tropical latitudes and the southern hemisphere, where birds are often territorial all year round (Odom et al. 2014), and in species with similar sex roles (e.g.

showing similar bi-parental care effort, Odom et al. 2021, Odom et al. 2014). Further, female song is often present in species with sexual monomorphism: therefore, complex female song is often mistakenly attributed to males, so is often overlooked (Langmore 1998, Webb et al. 2016).

Because song often serves a signal of quality or competitive ability in both sexes, but may serve different functions, the relative effects of different stressors on song may vary according to sex. For example, if male song is more complex than the females for a particular species, it may be that male song is more vulnerable to a decrease in quality associated with substantial stress. However, if songs are highly shared or equally complex, similar effects of stress may be expected for both sexes.

Finally, the most well-studied effects of stress on song have been in closed-end learners, which crystallise their songs before maturity. However, many species, such as the starling (*Sturnus vulgaris*), are open-ended learners and continue to learn new songs throughout their life (Chaiken and Böhner 2007). The effects of stress on learning are likely to persist into adulthood in open-ended learners – while in closed-ended learners, those effects may be restricted to the juvenile stage. Very little is known about how adult song performance is affected by short-term stress in open-ended learners, despite the evidence showing that chronic stress affects song.

1.2 Stress reactivity and personality

Stress is ubiquitous, and responding appropriately to stressors is essential for any animal's survival (Sapolsky et al. 2000). Responses to stressors are caused by a cascade of physiological changes, driven by the HPA axis, wherein the hypothalamus releases corticotrophin-releasing hormone, which regulates glucocorticoids, including CORT (Ellis et al. 2006). The changes through the HPA axis drive physiological activity and, thereby, behaviour, allowing organisms to respond appropriately to perceived threats or stressors (Smith and Vale 2006). These responses include increased blood flow and energy mobilisation, enabling behavioural responses such as triggering alarm calls and initiating flight (Sapolsky et al. 2000).

Since responses to stressors (e.g. flight response) are so energetically costly (Sapolsky et al. 2000), high levels of stress deplete energy reserves in birds and impair their ability to respond adequately to further stressors (Blas 2015, Noguera et al. 2017, Partecke et al. 2006). High

stress levels over long periods (chronic stress) also result in oxidative damage to proteins and DNA and impair condition-dependent signals, including songs (Alonso-Alvarez et al. 2007, Buchanan 2000). The effects of chronic stress can include a reduced energy response to stimuli, immune system suppression, neuronal cell death, muscle wastage, growth suppression and suppressed reproductive function (Sapolsky et al. 2000).

Responding appropriately to stressors strongly influences birds' survival and fitness and is under strong selection (Blas et al. 2007). A response must suit the situation; for instance, an underreaction and lack of response after perceiving a predator may result in predation, whereas an overreaction to a non-dangerous individual would be physiologically costly and wasteful (Cockrem 2013). Therefore, depending on the environmental context, birds with solid and rapid but appropriate responses to stressors in many circumstances can be an indicator of good quality (Buchanan 2000).

Individuals vary in their responses to stressful situations and, in doing so, have specific stress personalities. However, there is no single optimal stress response, as selection upon stress response is dependent on many factors, including ecological context (Bonier and Martin 2016); environmental predictability is particularly vital in the evolution of stress responses (Taborsky et al. 2021). For example, reactive personalities are beneficial in unpredictable environments, and proactive, less fearful individuals are well suited to constant conditions (Cockrem 2013). Further, the relative benefits of behavioural tendencies such as sociability, vigilance and freezing vary depending on resource availability (Montiglio et al. 2018). These benefits can also differ between sexes due to different requirements; for example, in great tits (*Parus major*), fast exploring females and slow exploring males had higher survival rates and more recruits in resource-poor years, and vice versa (Dingemanse et al. 2004). Great tits even disperse to areas where their personality is better suited to the local conditions (Mouchet et al. 2021). The expected benefits of stress reactivity variation can include energy conservation, beneficially changing reproductive timing, optimising offspring size or begging rates, and regulating the behaviour toward conspecifics (Breuner et al. 2008). Evidence suggests that these relationships are often very context-dependent, as selection pressures often fluctuate among different species; for example, glucocorticoid implants benefitted indirect fitness of the white-crowned sparrow (*Zonotrichia leucophrys*) but decreased indirect fitness in the pied flycatcher (*Ficedula hypoeuca*) (Breuner et al. 2008).

Stress personality is commonly estimated using three different methods. The first method involves observing the frequency or duration of certain behaviours throughout the day to pinpoint 'behavioural tendencies' (Konecna et al. 2008, Reale et al. 2007, Sinn and Moltischniowskyj 2005). This method can be ambiguous regarding the underlying assumptions of what that measured behaviour can tell us about the individual and can be more subjective in less familiar taxa, so it is primarily used in domestic animals or primate studies (Koski 2011). In particular, measuring 'behavioural boldness' is often criticised because it has been used to describe reactions to threats, dominance, confidence, and inquisitive tendencies (Carter et al. 2013, Roche et al. 2016). Therefore, the term 'boldness' also often arises in the second method of estimating stress personality by measuring neophobia. This method describes neophobic individuals are more reactive and cautious, and vice versa, in the context of unfamiliar environments or objects (Baugh et al. 2017b, Costa et al. 2020). The third method of estimating stress personality directly measures physiological stress and is defined by an individual's HPA response to stress, measuring the peak in glucocorticoids, whereby more reactive individuals have higher hormone increases (Cockrem 2007, Rensel and Schoech 2011). Most studies incorporate some combination of these measurements (Baugh et al. 2017a, Dingemanse et al. 2010a, Dingemanse et al. 2010b, Neave et al. 2020, O'Dea et al. 2020). To minimise subjectivity, researchers should consider using a combination of tests, test for correlations, and think critically regarding whether they are testing for the same or different traits and whether the trait in question is ecologically relevant or anthropomorphised (Carter et al. 2013, Koski 2011).

Research has provided good evidence that individual birds' hormonal reactivity can affect individual life-history trade-offs and influence individual fitness (Cain et al. 2016). For example, individual male and female dark-eyed juncos (*Junco hyemalis*) that produced more testosterone in response to gonadotropin-releasing hormone (GnRH) were more aggressive to simulated intruders (Cain et al. 2016). Similar relationships have been observed between physiological and behavioural reactivity when responding to stressors, with some birds being more or less reactive than others (Baugh et al. 2017b, Cockrem 2007, 2013). Exploratory tendencies in great tits (*Parus major*) have been linked to physiological responsiveness (Baugh et al. 2017a), and when in urban environments, to anthropogenic noise sensitivity (Grunst et al. 2021).

Stress can be measured in various ways, usually indicated by the measurement of CORT. Measuring CORT levels from blood plasma is the most common technique used and indicates an individual's current stress level (Romero and Reed 2005). However, it is also the most invasive method of measuring stress (Fairhurst et al. 2013), and it is very time-sensitive; CORT generally begins increasing within 3 minutes of capture (Romero and Reed 2005). A relatively new, non-invasive technique measures CORT levels in feathers. Since CORT is deposited throughout the period a feather grows, any substantial stress during that period is reflected in the CORT incorporated in the feather (Jenni-Eiermann et al. 2015). Because this method integrates circulating CORT levels over a protracted period (weeks), it may be a better indicator of the overall CORT profile than plasma CORT – which reflects the immediate stress levels (Romero and Reed 2005). Another non-invasive method is measuring CORT levels in faecal samples, which reflect individual stress levels from the previous two to four hours (Hansen et al. 2016), providing an easily accessible means of assessing CORT levels, averaged throughout that time (Cyr and Romero 2008, Xie and McWhorter 2021). These non-invasive measures have already been effective in predicting significant life events. For example, high feather and faecal CORT measures in harlequin ducks (*Histrionicus histrionicus*) indicated that reproduction was likely to be deferred (Hansen et al. 2016).

An even newer method builds on the physiological response to increased CORT levels. As the bird becomes stressed, its CORT increases through the HPA axis, increasing blood flow to the body's core and its core temperature. This change has been measured using thermal cameras in wild blue tits (*Cyanistes caeruleus*, Herborn et al. 2015, Jerem et al. 2018, Jerem et al. 2019, McCafferty 2013). Because the eye is highly vascularized and not covered in feathers, temperature changes in the eye are the most reflective of core temperatures (Jerem et al. 2019). Eye temperature measurements reflect relative stress in glucocorticoid levels, which correlates with individual quality proxies such as energy reserves, revealing chronic stress (Herborn et al. 2018, Jerem et al. 2018). These non-invasive methods provide reliable ways to measure baseline stress without causing the bird further stress through disturbance. These methods also indicate an individual's stress levels during different periods, such as immediate (eye temperature changes), within three to four-hour window (faecal), or during critical moult phases (feather) (Jenni-Eiermann et al. 2015).

1.3 My Aims

As shown above, birdsong plays a critical role in bird ecology, and long-term stress can significantly affect song quality. Current evidence suggests that stress physiology is likely linked to song quality and performance, but no study has looked at them together. Here I examine how measures of behaviour and reactivity to stress are related to song complexity and whether there are sex differences in those relationships. I aim to identify which non-invasive stress measurement technique most accurately predicts song complexity. The relationship between an individual's phenotypic (behavioural, thermal, faecal CORT) response to short-term stress and song complexity has not been investigated previously, and it may highlight how stress reactivity is related to individual quality.

This study examines the relationship between short-term stress and song performance in adult birds of both sexes. Specifically, I will address three inter-related questions:

1. How does short-term stress affect song complexity in adults?
2. Do the effects of short-term stress differ among individuals with different stress reactivities?
3. Do the sexes differ in their relationships between stress and song performance?

To address these questions, I will use behavioural responses, and two measurements of physiological stress, faecal CORT measurements and validate a new, non-invasive method of measuring stress using thermal imaging cameras to measure increases in body temperature, using captive common myna (*Acridotheres tristis*) – a species with pronounced and elaborate songs in both sexes.

To address these aims, I will: 1) determine whether eye temperature is an effective non-invasive method to measure stress in this species accurately; 2) identify the study subjects' stress reactivities using behavioural tests; 3) examine how individual stress reactivity relates to behaviour in baseline and elevated stress contexts; and 4) examine the relationships between short-term stress and song complexity, depending on sex and individual reactivity.

I predict that: 1) temperature will accurately correlate to an individual's stress reactivity and will increase after experimentally increasing stress; 2) subjects handling stress and neophobia

will be correlated in individuals; 3) behaviours that indicate stress, such as preening and movement, will increase in response to short term stress, and be displayed more frequently in reactive individuals; 4) songs will be complex in both sexes, but males will have a more extensive repertoire, higher singing activity and more complex features, and that less reactive individuals will have more complex songs and less singing activity; and 5) CORT treatment will increase singing activity but not affect song complexity.

2. Materials and Methods

2.1 Animals and housing

Study species

Common mynas (*A. tristis*) are an ideal species for investigating relationships between reactivity and song complexity. Mynas produce complex songs in both sexes, used for mate and conspecific communication, and they also exhibit vocal mimicry (Ingraham 2015, Lermite et al. 2017). Performing mimicry indicates that mynas continue to learn songs throughout their lifetime, thus they are likely open-ended learners similar to starlings (*Sturnus vulgaris*), their close relatives (Chaiken and Böhner 2007). The common myna has a global distribution due to introductions and persists in a variety of habitats and eats a wide array of food types (Wilson 2013). This tolerance to different environmental contexts, and their diet flexibility, has contributed to its invasiveness around the globe (Lowe et al. 2000, Magory Cohen et al. 2019). Mynas also have a high tolerance to captivity, as they are often kept as pets (Hart et al. 2020). The adaptability and robustness of myna makes them an ideal model species. All capture, restraint, and manipulation of myna was done under approval by the University of Auckland Animal Ethics Committee (Approval No. AEC2557).

Capture and housing

Thirteen common mynas were caught in September 2020 within the Auckland region. Some birds were obtained as live bycatch in an ongoing pest control operation. Otherwise, birds were caught as a part of a larger research project, using PeeGee traps in dry, sheltered areas on private properties. Traps were baited with dog food pellets and then left open for up to a week before capture to encourage neophobic birds to enter the trap. Myna song playback and olfactory baits (dog food pellets) were used to attract the birds. The traps were monitored every few hours (3-5h), with trapped birds having the space to exhibit normal behaviours and food and water available *ad libitum*. Non-target species were immediately released. The birds were transported in individual cages with non-slip surfaces, covered by an opaque fabric containing food and water. The research team transported the birds to a restricted-access lab at the University of Auckland, usually within approximately 20-30 minutes, but not exceeding one hour.

After capture, the research team took morphometric measurements (wing, tail, tarsus and bill lengths), photos and blood samples from all individual birds. The birds were weighed in a small opaque cloth bag with a Pesola® spring scale in the lab. Each bird was marked using different colour leg band combinations for individual identification during experiments. Blood samples, no more than 1% of total body weight, were collected using a brachial venipuncture with a 29-gauge sterile needle. Blood samples were sent to a molecular diagnostics lab to confirm sexing, as mynas are not strongly sexually dimorphic (Wilson 2013).

Mynas were individually housed in cages measuring 103cm x 45cm x 60cm, with room to stretch and fly to perches (Duncan 1981). The cages were attached to the top of the benches, allowing the birds to see and hear each other visually. Individuals were fed softened dog food pellets once a day and mealworms or sliced fruits once a week. Water was available *ad libidum*. Birds were allowed three weeks to acclimatise prior to experimentation. The lab had a day:night cycle of 12h:12h, was temperature-controlled at $23 \pm 1^\circ\text{C}$, soundproofed, and had a privacy shield for minimising disturbance to the birds when people entered the lab. The lab was fitted with four ceiling-mounted security cameras, capturing the activity of all birds 24/7. I placed a Bioacoustic Recorder (BAR; Frontier Labs, Australia) in the lab to record all vocalisations occurring between 0600h and 1800h. During the eight months in which the birds were being housed for the experiment, I (and others) took blood samples and weighed the birds twice (in addition to initial measurements) to monitor their health.

2.2 Neophobia experimental setup and procedure

To measure individual reactivity and neophobia, I created four copies of three types of novel objects in the form of small food containers. The three different novel objects varied in shape, colour and reflectiveness (Fig. 1), as recommended by Greggor et al. (2015). I designed the containers to ensure they appeared novel and unique, rather than eliciting fear or attraction. Although fear or attraction responses depend on previous experience (e.g. aposematic colouration), I ensured the containers had no sharp aspects and avoided red and black colouration, which could be perceived as aposematic colouration (Greggor et al. 2015). The control container was an orange terracotta food plate (15cm diameter), which was also used to provide the birds' maintenance diet daily. Before trials, I placed separators between cages, so that neighbouring birds were not visible to each other during the experiment. Observers were also not visible to the birds. I did not deprive the birds of food for this experiment,

although morning food provisioning was delayed by one hour while the experiment was conducted and no additional food was provided during the experiment. When placed inside the cage, each container (including the control container) contained one live mealworm.

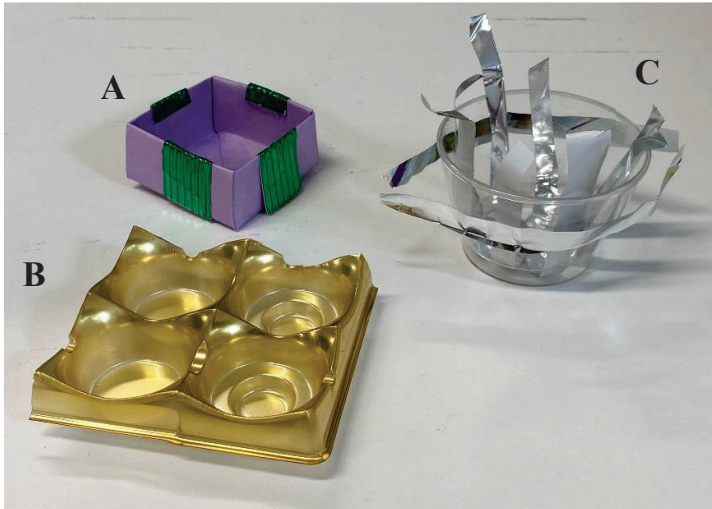


Figure 1. Image of the three novel containers. Container A, used for trial 1 (N1). Purple paper origami box, with reflective green corrugated cardboard attached to the outside, measuring 5.5cm x 5.5cm x 3cm. Container B, used for trial 2 (N2). Reflective gold plastic tray measuring 9.7cm x 9.7cm x 4cm. Container C, used for trial 3 (N3). Clear plastic cup, with attached reflective soft plastic strips, measuring 5cm x 7cm.

Testing birds in pairs (and one group of three), I placed one copy of Container A, each containing one mealworm, on the floor of each bird's cage simultaneously. As soon as I was out of the bird's sight behind the privacy screen, I recorded each bird's time to eat the mealworm from the container. Birds were given a maximum of twenty minutes to respond to the containers before the container was removed. This procedure was repeated for each pair of birds, in a randomised order, for the three novel container types and the control container. For one of the containers, Container C, the mealworms became stuck underneath the plastic strands, so the timer was stopped when the bird attempted to eat the mealworm, rather than when they succeeded in eating it.

2.3 CORT experimental procedure

Baseline measurements

I took baseline measurements of vocalisations, behaviour, temperature and faecal CORT for each bird. Baseline measurements are used as a control to assess levels of critical metrics

during periods when the birds' stress has not been experimentally induced. I selected two full baseline days for taking these measurements; birds were not disturbed on the day of the baseline measurements or the previous day.

CORT treatments

To elevate CORT levels without directly stressing the birds, I experimentally elevated CORT in the birds over two days by feeding the birds mealworms injected with CORT (control, low, high). These experimental periods, where the birds were induced into a state of elevated stress, are referred to as elevated states. Experimentation hours occurred between 0900h and 1500h when natural daily CORT levels were at a minimum and relatively stable (Rich and Romero 2001). Dose sizes were determined based on previous studies on similarly sized birds (Breuner et al. 1998).

Frozen mealworms injected in the ventral side of the abdomen, on the second segment from the end (Breuner et al. 1998). Any mealworms that collapsed upon injection and leaked were replaced. Mealworms were presented to the birds on a 1cm² slice of banana to ensure any oozing liquid was ingested (myna readily eat bananas). One mealworm and banana combination was placed on usual food plates on the floor of each cage simultaneously. The injection solutions were: Control - 20ul of Dimethyl sulfoxide (vehicle - DMSO) without CORT; low CORT - 10ug of crystalline CORT suspended in 20ul of DMSO; or high CORT - 20ug of CORT suspended in 20ul of DMSO. The DMSO is an inactive ingredient used as an efficient solvent for the crystalline CORT treatments to inject into mealworms (Breuner et al. 1998)

2.4 Measurements

Vocalisation analyses

Vocalisations were recorded during baseline and elevated CORT using the four ceiling-mounted cameras and the Bioacoustic Recorder (BAR, Frontier labs, Australia). I individually selected each myna vocalisation using Raven Pro version 1.6.1. (Charif et al. 2010). I identified the singer by aligning the vocalisation time with the time individuals were observed singing (bill open) on the video footage using SmartViewer (Hanwha Techwin America 2020). On 'control days (baseline stress),' vocalisations during the first five minutes of each half-hour, between 0600h and 1800h, were analysed. Five-minute periods were shifted by 15 minutes if anyone entered the lab, e.g. to feed the bird. For periods of experimentally 'elevated' stress, vocalisations were analysed in five-minute sections for the

entire 30 minute period immediately after CORT was consumed. CORT levels usually return to baseline levels within 30 minutes of elevation (Breuner et al. 1998).

Calls were defined as short vocalisations ranging in frequency from approximately 0.5 to 3Hz. These are usually performed with a closed bill. I excluded calls from the complex vocalisation analysis as I could not identify which individual produced them from the video footage. Songs included all other vocalisations, which were more complex, and varied in frequency and length (Fig. 2; Fig. 3). I selected a range of robust song metrics that minimise typical issues, such as distance from the microphone, reverberation and human subjectivity (Table 1; Charif et al. 2010).

Song analysis was completed on Raven Pro version 1.6.1. (Charif et al. 2010) and web-based software Koe (Fukuzawa et al. 2020). Raven Pro version 1.6.1 was used to make song selections, including songs where only one bird was singing at a time (excluding calculations of total singing time). Koe was used to classify syllable types and their parameters using its automated similarity feature, using principal component analysis (PCA) (Fukuzawa et al. 2020). Syllables were roughly classified into six categories (Fig. 3). I created the song metric ‘syllable score’ (Table 1) by scoring each syllable type from 1-6 (Fig. 3). Scores increased based on which were deemed more difficult and thus more likely to indicate quality, such as song rate, bandwidth and fast sweeps in frequency (Goller 2022, Podos et al. 2016). The song metrics I chose for analysis were parameters that are most likely to capture song complexity and have been used widely in a variety of studies investigating song complexity and individual quality (Table 1; Badyaev et al. 2002, Benedict and Najar 2019, Brandes 2008, Cain et al. 2015, Hill and Pawley 2019, Ríos-Chelén et al. 2016).

Table 1. Song measurements that were used in song complexity analyses and their definitions. Song measurements marked ‘^{Koe}’ indicates they were automatically calculated by the programme Koe (Fukuzawa et al. 2020). Other song measurements regarding syllables were extracted in Koe but calculated manually.

| Song measurement | Song unit measured | Definition |
|---------------------------------------|--------------------|------------------------------------------------------------------------------------------------------------|
| Syllable rate (syllables per second) | Song selection* | Number of selected syllables in song selection divided by song selection length (seconds). |
| Syllable diversity | Song selection* | The number of different syllables in a song selection divided by the total number of syllables. |
| Syllable switches | Song selection* | The number of times the syllable switches type in the song selection. |
| Syllable score | Song selection* | The summed score of each syllable (Fig. 3) divided by the total number of syllables in the song selection. |
| Bandwidth (Hz) ^{Koe} | Syllable | The magnitude weighted mean of the highest and lowest frequencies in the syllable. |
| Spectral flux ^{Koe} | Syllable | A measure of changes in the shape of the syllable over time. |
| Maximum frequency (Hz) ^{Koe} | Syllable | The frequency at which the maximum power (dB) occurs in the syllable. |

* ‘Song selection’ is used instead of ‘song’ as the section analysed is not necessarily an entire ‘song’ as some selections are cut off due to multiple birds singing at once.

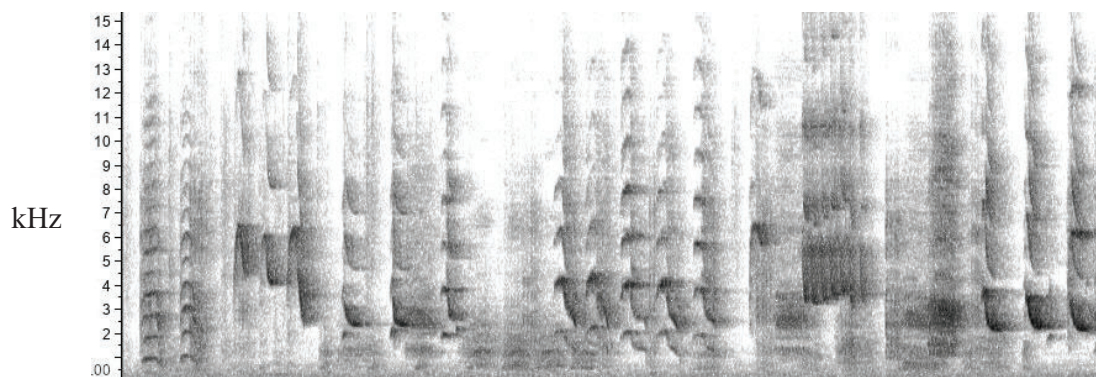


Figure 2. Spectrogram of common myna (*Acridotheres tristis*) song, kHz over time. Taken from Raven Pro version 1.6.1.

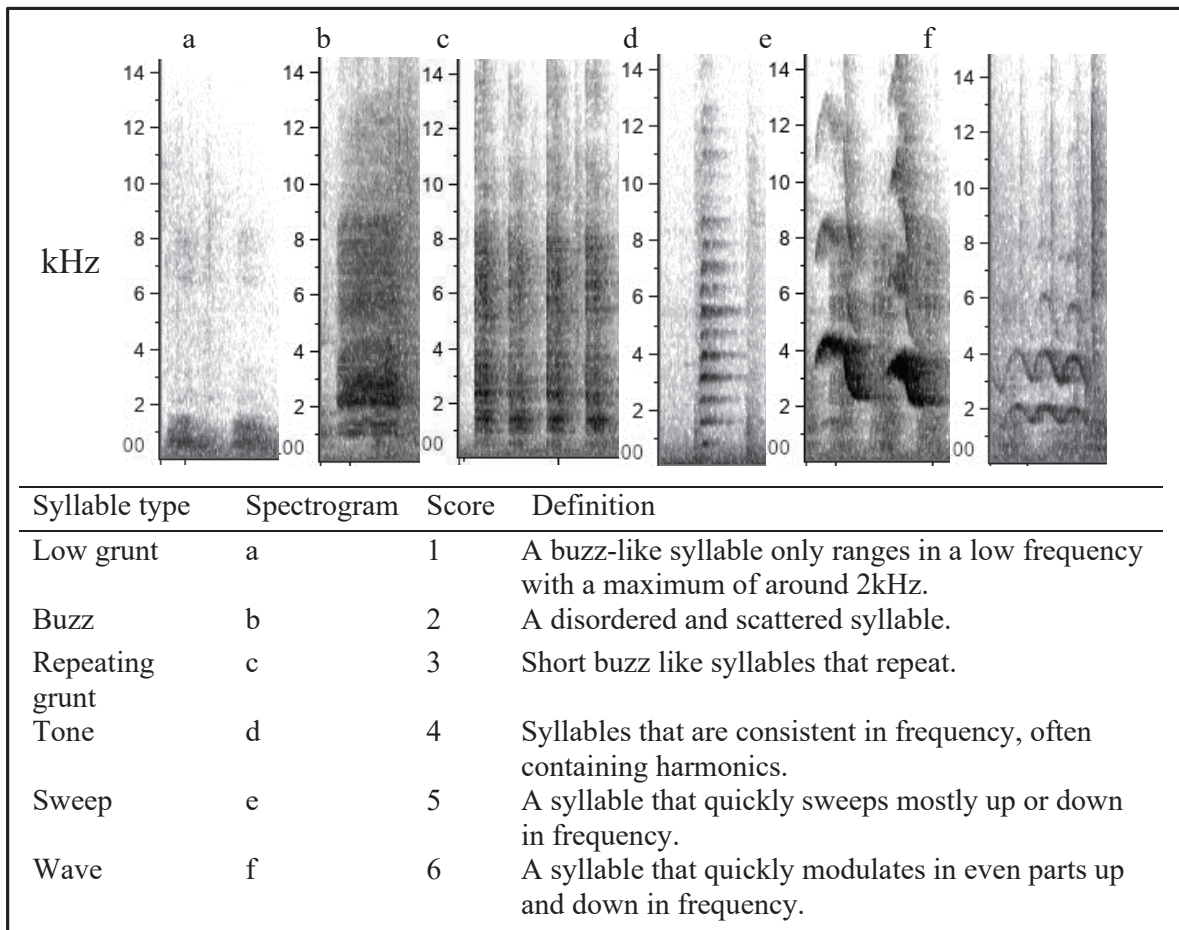


Figure 3. Spectrogram of typical common myna (*Acridotheres tristis*) syllable types, and definitions based on previous research. Arbitrary scores are given by their relative complexity, based on previous research. Spectrogram is kHz over time. Spectrogram was produced on Raven Pro version 1.6.1.

Behaviour analyses

Behaviour was scored using the software BORIS version 7.10.5 (Friard and Gamba 2016) using video footage captured from installed cameras through SmartViewer (Hanwha Techwin America 2020). Behaviours analysed were the neophobia trials and the activity of behaviours during the individual 30-minute CORT elevation experiment. Recordings were divided into five-minute periods for analysis. I chose point (frequency) and state (duration) behaviours that are indicative of stress, anxiety or thermoregulation (Table 2).

Table 2. Ethogram of myna behaviours recorded from video footage using BORIS version 7.10.5 (Friard and Gamba 2016) and Smartviewer (Hanwha Techwin America 2020). Point behaviours were recorded as events (frequency), while state behaviours were recorded using time interval recording (duration).

| Behaviour | Definition | Justification for including behaviour |
|-------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <u>Point behaviours</u> | | |
| Walk | Bird takes a minimum of two steps on the floor, shuffling on perch or climbing along the cage wall. | Movement often increases substantially with stress or anxiety in birds (Astheimer et al. 1992, Breuner et al. 1998, Breuner and Wingfield 2000). |
| Moves | Bird moves from the surface where they are currently standing. Movement by jumping from the perch to the floor or any flying behaviour. | |
| Ruffle feathers | The bird shakes and ruffles its whole body's feathers up. Not including a slight ruffle while preening. | Ruffling feathers increases highly above baseline with stress and is often used to downregulate temperature (Bauer et al. 2011, de Bruijn and Romero 2011, Lattin et al. 2017). |
| Drink | The bird puts its head into the water bottle. | Increased drinking is often used to downregulate temperature in birds (Hafez 1964). |
| <u>State behaviours</u> | | |
| Newspaper rustle | Bird rips, lifts or moves newspapers on the cage floor with its bill or foraging for fallen food within newspaper on the floor. | Ripping or searching in cage covering newspaper is attributed to bird's foraging behaviour, which increases with stress or anxiety above baseline levels (Breuner and Wingfield 2000, Hinde 1953). |
| Cleaning beak | The bird wipes either side of its beak on any surface. | Beak wiping or cleaning increases substantially when stressed or anxious (Bauer et al. 2011, Lattin et al. 2017). |
| Panting | The bird is breathing quickly and holds bill open. | Birds often use open beak panting to downregulate temperature (Hafez 1964). |
| Preening | The bird grooms feathers on its body, with its bill or feet. If the bird stops, it is no longer than five seconds. | Preening more frequently than during baseline is associated with stress (Lattin et al. 2017) |

During the routine health monitoring, the bird handler scored the birds' behaviour and temperament in hand from 1-5, following a similar method to Brommer and Klueen (2012) (Table 3). Criteria for the stress rating included grabbing with their feet, biting and alarm

calling (Table 3). This procedure was undertaken on two separate days, providing two measurements of handling stress for each bird.

Table 3. Scoring for handling stress rating.

| Stress rating | Behaviours displayed by the bird while in hand. |
|---------------|---------------------------------------------------------------------------------------------------|
| 1 | The bird shows minimal signs of stress, some grabbing with feet but showing no apparent distress. |
| 2 | Mild levels of grabbing or biting. Not alarm calling. |
| 3 | The bird grabs or bites a lot and shows mild wriggling or kicking. The bird is not alarm calling. |
| 4 | The bird is wriggling and kicking a lot. The bird may alarm call. |
| 5 | The bird is alarm calling, wriggling and kicking a lot. The bird may be shivering. |

To create a composite score of stress reactivity for each individual, I combined the retrieval times from the neophobia trials and handling stress scores in a principal component analysis (PCA). Principal component one (PC1) from this PCA accounted for 59.9% of the variation observed, and I used this as a score of stress reactivity.

Temperature measurements

Eye temperature measurements at elevated and baseline levels were taken remotely in the lab using a thermal camera (FLIR T620 camera, Teledyne FLIR®, USA) with an infrared resolution of 640 x 480. The camera was fixed to a tripod 1m in front of the cage, where the lens was level with the cage's floor, where the bird was fed. The camera on the tripod was set up in the lab 20 minutes prior to taking measurements on any day. Thermal images were only used in the analyses if the camera was in focus and the bird's eye was facing the camera (Fig. 4), as these criteria are essential for an accurate temperature reading (Charif et al. 2010, Jerem et al. 2018). All thermal images were taken between 0900h and 1500h. The birds were photographed in randomised order on each temperature measurement day.

I measured temperature in CORT elevated state over two days, five days after measuring CORT elevated vocalisations, so that vocalisations would not be affected when I entered the room to move the camera tripod to photograph each bird. On these days, the same experimental procedure was followed to elevate each bird's CORT individually, but using the high CORT dose only. I provided each bird with a high CORT mealworm and banana combination individually while I photographed them remotely. To encourage them to remain

on the cage floor, and therefore in the camera frame, I removed the top perches from each cage 20 minutes before mealworm provision. I started taking photos five minutes before giving them CORT mealworms to establish their current baseline temperature and continued for 25 minutes after the bird ingested the mealworm when CORT is expected to be back to baseline levels (Breuner et al. 1998).

Baseline temperature measurements were taken on four different days, separate from the days measuring baseline vocalisation and behaviour. I took approximately 20-30 thermal photos over 10 minutes for each bird, allowing five minutes between birds after moving the tripod. FLIR tools version 6.4 was used to select the best thermal images taken to use for analysis. I averaged the eye temperature measurement for each minute for each individual for analysis.

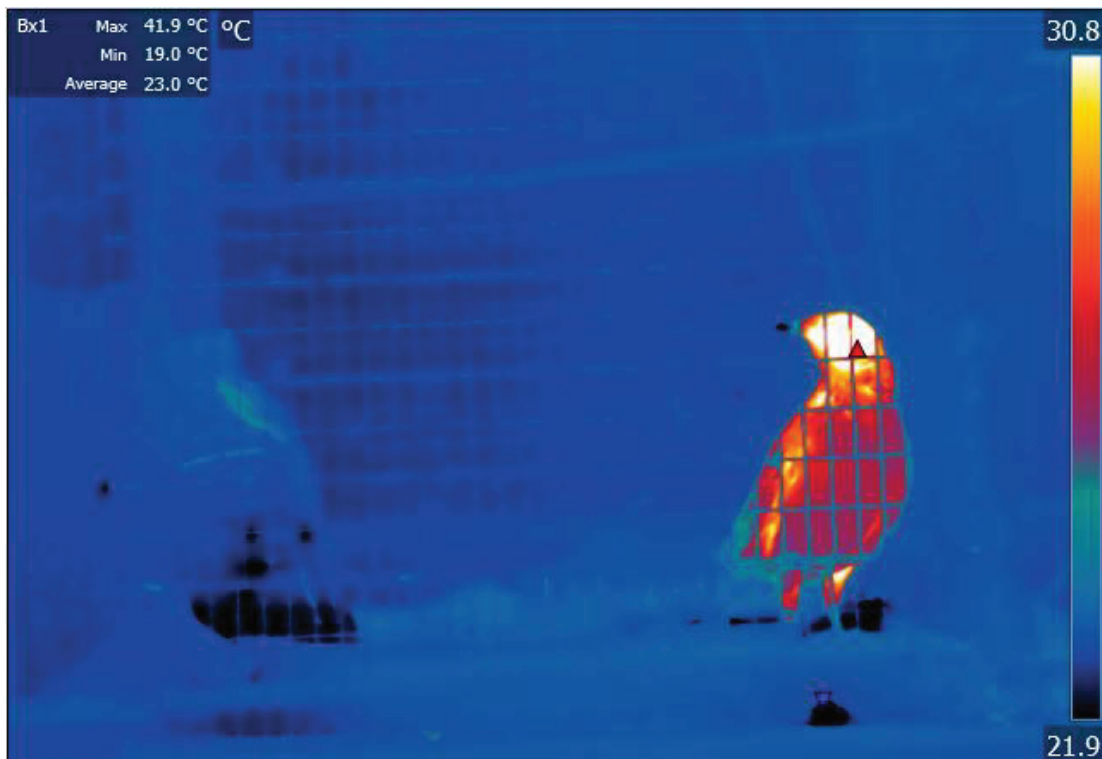


Figure 4. Thermal image of an individual myna (B1) during baseline stress on 29/04/2021. Temperature (°C) is indicated by colour, where the red arrow indicates the maximum temperature in the image, which is the myna's eye (41.0 °C). Photo (FLIR T620 camera, Teledyne FLIR®, USA).

Faecal CORT assays

I collected faecal samples on two baseline days and on the three experimental CORT days when the birds were fed experimental mealworms (control, low, high). I placed the silicone baking paper sheets in the cages 30 minutes before CORT mealworms were provided since placing the sheets is relatively invasive. Sheets were placed on each bird's cage floor at 0900h and experimental mealworms were provided at 0930h. Sheets were removed at 1400h on baseline days and 1130h on experimental days, as CORT metabolites take 1-2 hours to appear in faeces (Goymann et al. 2002). Birds were expected to have passed the CORT mealworms well within this time, given mynas' gut passage rate is likely comparable to closely related starlings, with a gut passage range of 10 to 60 minutes (Levey and Karasov 1994). Faecal samples were then collected from the sheets into eppendorf tubes and frozen within two hours to avoid degradation of metabolites (Palme 2019, Touma and Palme 2005).

Faecal CORT hormone analysis

Validations were completed before samples were run to identify the appropriate dilution factor and extraction concentrations (Palme 2019, Touma and Palme 2005). For CORT validations, I pooled homogenised dry faecal samples from the 13 birds and extracted them in 60% methanol (Palme et al. 2013). I performed serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. This serial dilution was compared to the standard curve, testing for parallelism. I then created a series of spiked recoveries using replicates of a sample diluted to 1:128 and spiked with the five standards alongside the standard curve. Validations showed good parallelism and high spike-recovery values (recovered 101.31%, stdev = 0.663pg/ml).

Extracted samples were assayed using an Enzo LifeSciences corticosterone-acetylcholinesterase (AChE) ELISA kit to detect faecal glucocorticoid metabolites (FGMs) in our samples. Enzyme immunoassays (EIAs) are the most effective detection system for faecal metabolites in birds (Bienboire-Frosini et al. 2018).

Faecal samples were frozen until processing. I lyophilised the frozen faecal samples using a freeze-dryer (Alpha 2-4 LSC basic, Martin Christ®, Germany), then crushed the samples individually to a fine powder using a pestle and mortar. From each homogenised dried faecal sample, I subsampled 0.1g, weighed on a precision scale (AE166 DeltaRange®, Mettler, USA) and then suspended in 1 ml of 80% methanol for extraction of metabolites. I vortexed each sample for 30 seconds, then placed the samples in an orbital shaker for 30 minutes at 420 rpm. Samples were then centrifuged for 20 minutes at 2,500 rpm. I transferred the

supernatant to new tubes and evaporated the methanol from the samples under a gentle stream of nitrogen at 45°C. Once all the methanol was evaporated, the samples were stored in the fridge until resuspension.

I then resuspended each sample tube in 25ul of 100% ethanol, then vortexed for 30 seconds. 150ul of assay buffer was added to each sample tube, then vortexed for 30 seconds. Samples were further diluted by transferring 10ul of supernatant to a clear tube, and adding 1000ul of buffer to each, creating a dilution factor of 1:100. I ran all standards and samples in duplicate. I followed the ELISA kit protocol instructions for plating, creating a standard curve and control wells. Corticosterone concentration is measured through the light absorbance in each plate well. Plates were read with a microplate reader (Spectramax® iD3, Molecular Devices, USA). Faecal samples containing less than 0.1g were checked for disproportionately high concentrations (Goymann 2005).

2.5 Analysis

Using thermal imagery to measure stress

To test for an effect of CORT treatment (baseline/elevated) on eye temperature, I created a linear mixed model (LMM, family = Gaussian) (package 'lme4'), including individual as a random factor, due to repeated measures of the same individual. The initial model included the date the thermal image was taken, but no effect was found ($p > 0.20$), so it was excluded from the final model. Temperature was taken in 5-minute periods. To test whether temperature changed over time, following the ingestion of a CORT injected mealworm, I built a separate LMM with temperature as the response variable and 5-minute period and sex as predictor variables. Because significant effects were found between 5-minute periods, I used a post-hoc Tukey constraints test to identify differences between 5-minute periods.

To test whether metabolised CORT increased when the birds ingested the mealworms injected with CORT, I built a LMM with log-transformed faecal CORT concentration as the response variable and CORT treatment as the effect (baseline days, control, feeding 10ug and 20ug of CORT), including individual as a random factor. There were three extreme values (value $> 3 * SD$), so this model was run twice, including and excluding these values. Because significant effects were found between CORT treatments on faecal CORT concentrations when extreme values were excluded, I used a post-hoc Tukey constraints test to identify differences between the treatments.

I log-transformed faecal CORT concentration to test the relationship between eye temperature and faecal CORT concentration. I built a LMM with eye temperature as the response variable and logged faecal CORT concentration as the predictor variable, including the subject as a random factor.

Measuring stress reactivity

I built a Kaplan Meier survival model to identify whether there was consistency in mealworm retrieval times for the three different neophobia treatments (Fig. 1). To test whether handling response score was related to neophobia, I built a linear model (LM) to test the relationship between neophobia retrieval times as the response variable and handling stress response score as the predictor variable, including subject as a random factor. Stress reactivity score was defined using the first principal component in the PCA as previously mentioned. I tested whether sex affected stress reactivity score, using Welch's two sample t-test, as variance in stress reactivity between the sexes had equal variance, tested with a Levene's test.

Stress reactivity and measures of stress

To test the relationships between stress reactivity scores and measures of stress (faecal CORT and eye temperature), in baseline and elevated contexts, I created two sets of LMMs, including individual as a random factor in each. The first LMM tested the relationship between stress reactivity and eye temperature at elevated and baseline CORT, and the second for how stress reactivity related to logged faecal CORT at baseline and elevated stress. Temperature and log faecal CORT concentration were the response variables in each LMM, and the predictor variables in both included the interactions of CORT treatment (baseline/elevated), sex and stress reactivity (PC1). To make the log faecal CORT concentration model more parsimonious, I used backwards-stepwise removal to simplify the models, beginning with interactions and if the model had a lower AIC, ($\Delta AIC > 1$) (Hurvich and Tsai 1989), without a parameter, it was dropped.

Stress physiology and behaviour

To test for the effects of stress reactivity (PC1) and CORT treatment (elevated or baseline) on duration and frequency of behaviours, I built a set of GLMMs (family = Poisson). I focused behaviour types that described unique actions, so 'eating', 'cleaning beak' and 'walking' were excluded from further analyses, as these activities were correlated to 'drinking', 'preening' and 'moves position' respectively. Response variables tested by frequency were 'drinking', 'moves position' (flying or jumping down from perch) and 'ruffling feathers'.

Response variables tested by duration were ‘rustling newspaper’ and ‘preening’. The individual was included as a random effect in all GLMMs. I ran each model including the effect of interaction of treatment and sex, and if the model had a lower AIC score without it, it was dropped. One individual produced extreme values for some behaviours, so the models were run with and without this individual – both models are reported. To plot the difference in behavioural activity for both measurements, I calculated the difference in behavioural frequency or duration before and after the birds ingested a mealworm injected with 20ug of CORT, which indicates whether behaviour increased or decreased after CORT ingestion. Because these data were on different scales and had extreme values, measures were scaled before plotting.

The effects of stress on singing

To test whether singing activity throughout two baseline days was affected by stress reactivity or sex, I built a LM. Total singing activity was the response variable, and predictor variables included stress reactivity, sex(m/f) and day(1 and 2). To test whether singing activity over 2.5 hours was affected by CORT treatment, I built another LM. Total singing activity was the response variable, and interactions of stress reactivity, CORT treatment and sex were the predictor variables. Both LMs included subject as a random effect. Both models were also simplified using AIC values to exclude variables. If removing a variable increased the AIC of the model by >1, the variable was retained.

Testing for effects on song complexity

I built eight LMMs to test for relationships between song metrics and stress reactivity, CORT treatment, and sex. The response variables of each LMM were syllable rate, syllable diversity, syllable switches, syllable score, spectral flux, maximum frequency, and bandwidth. Predictor variables included the interactions of stress reactivity, sex (m/f) and treatment (baseline/elevated). Each model included the subject as a random effect.

To test which measure of stress is the best at explaining measures of song complexity, I built two sets of models, including LMMs, and a linear regression model. The first set of models tested songs during baseline treatment, and the second at elevated treatment. Each set had the response variables; syllable diversity, syllable switches, syllable score, spectral flux, maximum frequency, and total time singing. Total time singing had no repeat measures, so the model was a linear regression. Predictor variables of each model were stress reactivity, temperature and log faecal CORT, and the interaction of sex with each. To determine the best

models for each parameter, I used backwards-stepwise removal to simplify the models, beginning with interactions and removing variables where $P > 0.20$.

All statistical analyses were performed using R studio version 4.0.3. Graphs were produced using the *'ggplot2'* package, and tables were produced using the packages *'sjPlot'* and *'stargazer'*. PCAs were made using the package *'factoextra'*, and effect plots were made using the package *'effects'*. The Kaplan Meier model was made using the package *'survival'*.

All linear mixed models (LMM) and linear models (LMs) were made using the package *'lme4'* and *'lmerTest'* and run with a Gaussian distribution unless otherwise stated.

3. Results

3.1 Using thermal imagery to measure stress

I found strong evidence that ingestion of a CORT injected mealworm increased individual myna's core temperatures over time (Fig. 5, Fig. 6; Table 4). There was a distinct pattern in temperature response after CORT was ingested (Fig. 5), with a temperature increase in the first five minutes following CORT ingestion ($p < 0.001$). Following the initial increase in the first five minutes after mealworm ingestion, bird eye temperatures decreased. After 10mins, temperature was not significantly different from pre-ingestion baseline temperatures (Fig. 5, Table 4). There was considerable individual variation in temperature response after ingesting CORT (Fig. 6). Females tended to have slightly higher eye temperatures overall, although not significantly (estimate = 0.36, $P = 0.19$; Table 4).

Table 4. Summary of the linear model (LM) results testing the effect of the 5-minute period relative to ingesting a CORT injected mealworm on individual bird eye temperature and sex (m/f). The variance attributed to the random effects of Subject ID and the residual is presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold.

| <i>Predictors</i> | Temperature (°C) | | | |
|------------------------------------------------------|--------------------------|---------------|------------------|------------------|
| | <i>Estimates</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept – pre-treatment) | 40.76 | 40.34 – 41.18 | 190.18 | <0.001 |
| 0-5 minutes after CORT | 0.64 | 0.42 – 0.87 | 5.69 | <0.001 |
| 5-10 minutes after CORT | 0.25 | 0.02 – 0.47 | 2.15 | 0.031 |
| 10-15 minutes after CORT | 0.05 | -0.18 – 0.28 | 0.41 | 0.679 |
| 15-20 minutes after CORT | -0.14 | -0.37 – 0.09 | -1.19 | 0.233 |
| Sex [M] | -0.36 | -0.90 – 0.18 | -1.32 | 0.188 |
| Random Effects | | | | |
| Residual effect | 0.42 | | | |
| Individual effect | 0.23 | | | |
| Marginal R ² / Conditional R ² | 0.142 / 0.445 | | | |
| AIC | 666.645 | | | |

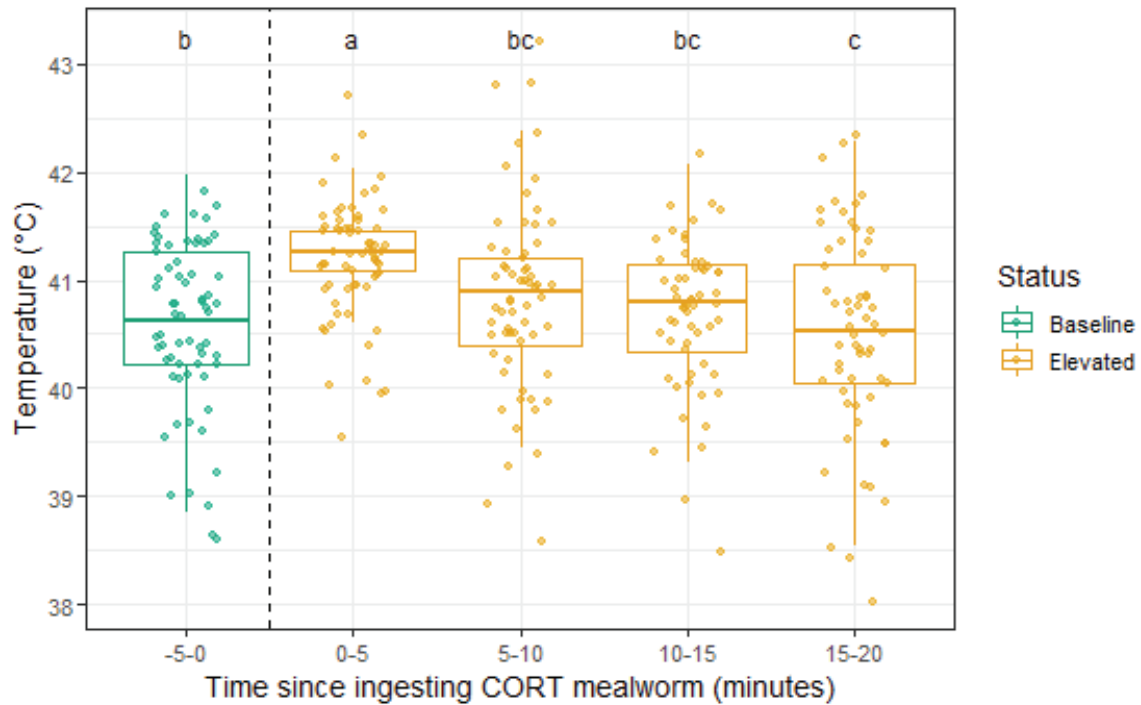


Figure 5. Boxplots of eye temperatures preceding (green = baseline) and following CORT mealworm ingestion (yellow = elevated), grouped into five minute periods. Dots indicate individual temperature measurements at each time point. The upper and lower whiskers of the boxplots indicate 75% and 25% quartiles $\pm 1.5 \times$ interquartile range. The box's upper and lower edges represent 75% and 25% quartiles, and the median is the thick line in the centre of the box. A linear mixed model (LMM) was used to test how 5-minute period affected temperature, with subjects as a random factor (Table 4). Tukey constraints test on the LMM was used to test differences between 5-minute periods. Lower case letters indicate significant differences ($P < 0.05$) between groups.

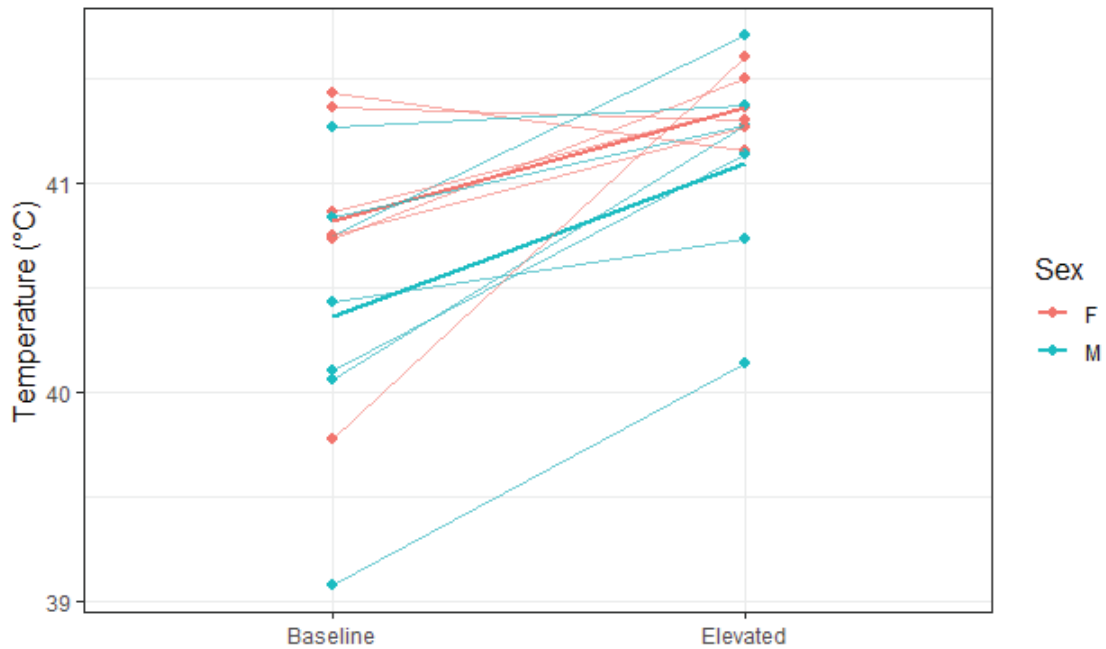


Figure 6. Variation in each individual's mean eye temperature at baseline (taken during the five minutes before ingesting CORT) and elevated (taken during the first five minutes following a mealworm injected with 20ug of CORT) stress levels. Individuals are indicated by thin lines connecting points, females (F) are indicated by red lines and males (M) indicated by blue lines; thick lines indicate averages for each sex. Females were slightly warmer overall, although not significantly (Table 4).

The LMs that tested for the relationships between faecal CORT concentration and CORT treatments, found that CORT increased after ingestion of treated mealworms. CORT concentration in faecal samples collected after birds received the high CORT treatment (ingesting a mealworm injected with 20ug CORT) was significantly higher than samples collected with control and low treatments (Table 5). When extreme values were excluded, the low CORT treatment (10ug CORT) faecal CORT concentration was significantly higher than the second baseline treatment, but not the control (Fig. 7, Table 5). Because the low CORT treatments were not significantly different from baselines and control, the low CORT treatment was excluded from further analyses.

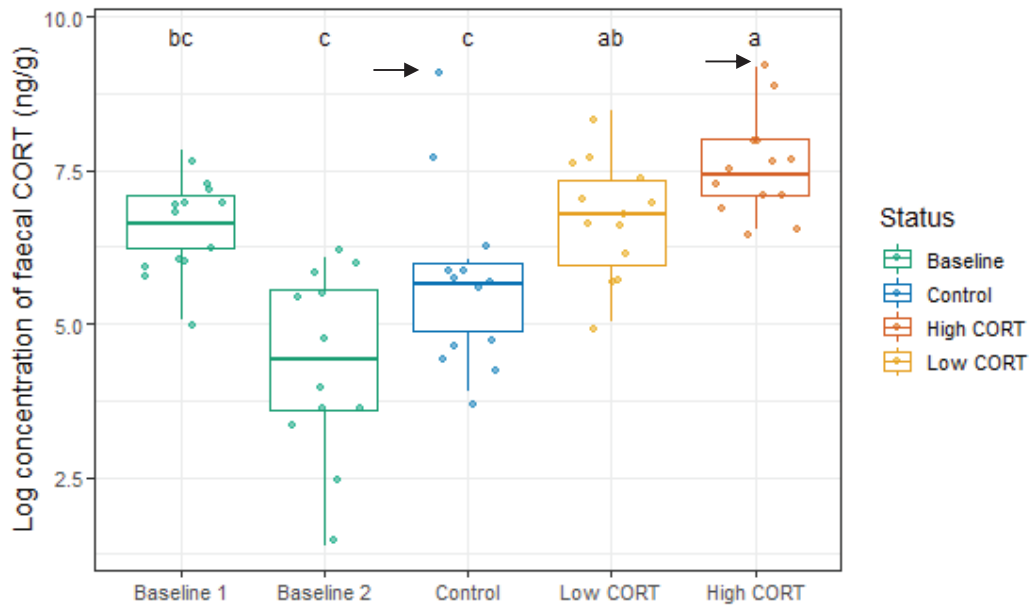


Figure 7. Boxplot displaying log faecal CORT concentration at each CORT treatment level. CORT treatment is indicated by colour. Green or baseline indicates no treatment; blue indicates the control treatment (DMSO only). Low and high CORT indicated treatments of ingesting mealworms injected with 10ug (yellow) and 20ug (red) of crystalline CORT dissolved in DMSO, respectively. Dots indicate each measurement of faecal CORT concentration. The upper and lower whiskers of the boxplots indicate the largest and smallest observations within 25% and 75% quartiles $\pm 1.5 \times$ interquartile range. The box's upper and lower edges represent 75% and 25% quartiles, and the median is the thick line in the centre of the box. Significance indicated by letters via, Tukey post-hoc test on LMM, excluding extreme values where $n > 3 \times SD$. Extreme values are included in the plot and are indicated with black arrows.

Table 5. Summary of linear models (LMs) results showing the effects of CORT treatment on faecal CORT concentration ng/g, with and without extreme values (value > 3 * SD). CORT treatment includes two days of baseline faecal CORT concentration (no treatment), control (ingestion of DMSO injected in mealworm), and 10ug and 20ug of CORT injected in a mealworm, respectively. The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold, marginal estimates where $P < 0.01$ are indicated in italics.

| Faecal CORT concentration (ng/g) | | | | | | | | |
|------------------------------------------------------|------------------------|---------------------|-----------|------------------|---------------------|--------------------|-----------|--------------|
| Predictors | Without extreme values | | | | With extreme values | | | |
| | Estimates | CI | Statistic | <i>p</i> | Estimates | CI | Statistic | <i>p</i> |
| (Intercept – Baseline 1) | 898.98 | 386.86 – 1411.11 | 3.44 | 0.001 | 898.98 | -93.54 – 1891.51 | 1.78 | <i>0.076</i> |
| Baseline 2 | -733.00 | - 1353.74 – -112.26 | -2.31 | 0.021 | -739.63 | -2159.51 – 680.25 | -1.02 | 0.307 |
| Control | -515.00 | - 1135.74 – 105.74 | -1.63 | 0.104 | 286.13 | -1104.58 – 1676.84 | 0.40 | 0.687 |
| 10ug CORT | 415.09 | -191.20 – 1021.37 | 1.34 | 0.180 | 415.09 | -975.62 – 1805.80 | 0.58 | 0.559 |
| 20ug CORT | 1113.31 | 492.57 – 1734.05 | 3.52 | <0.001 | 1687.10 | 296.39 – 3077.81 | 2.38 | 0.017 |
| Random Effects | | | | | | | | |
| Residual effect | 621971.31 | | | | 3272578.24 | | | |
| Individual effect | 265584.19 | | | | 61141.37 | | | |
| Observations | 62 | | | | 64 | | | |
| Marginal R ² / Conditional R ² | 0.329 / 0.530 | | | | 0.157 / 0.173 | | | |
| AIC | 962.032 | | | | 1080.298 | | | |

The LM testing the relationship between individual eye temperature and their faecal CORT concentration found a marginal positive relationship ($p < 0.001$, Fig. 8; Table 6), even though the variation was relatively high (95% CI 0.39 – 2.58). A high amount of variation was observed in temperature and faecal CORT concentration overall, with higher variation at baseline measures, but more consistent high readings at elevated CORT (Fig. 8).

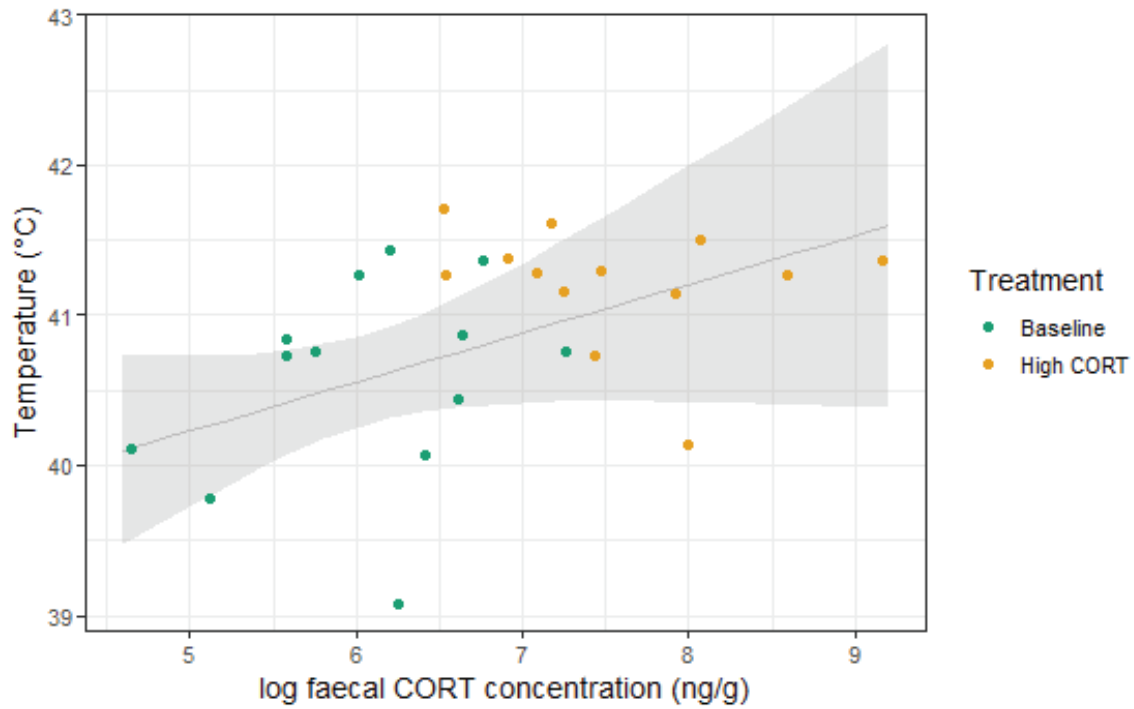


Figure 8. Linear model results showing the relationship between log-transformed faecal CORT concentration and eye temperature. Colour differences show CORT treatment, where baseline (green) indicates no treatment (baseline values were the averaged baseline measurements taken on two different days), and high CORT (yellow) indicates birds after ingesting a mealworm injected with 20ug of crystalline CORT. The linear regression line is shown in grey, conditional $R^2 = 0.67$. The grey band indicates 95% confidence intervals.

Table 6. Summary of the linear mixed model (LMM) results showing the relationship between faecal CORT concentration and individual eye temperature, including interactions of treatment (baseline/high CORT). The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold, marginal estimates where $P < 0.1$ are indicated in italics.

| <i>Predictors</i> | Temperature °C | | | |
|------------------------------------|-----------------------|---------------|------------------|------------------|
| | <i>Estimates</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept) | 38.60 | 36.30 – 40.90 | 32.89 | <0.001 |
| Log Faecal CORT | 0.33 | -0.05 – 0.70 | 1.70 | <i>0.090</i> |
| Treatment [High] | 2.95 | -0.28 – 6.17 | 1.79 | <i>0.073</i> |
| Log Faecal CORT * Treatment [High] | -0.37 | -0.84 – 0.10 | -1.53 | 0.126 |
| Random Effects | | | | |
| σ^2 | 0.15 | | | |
| τ_{00} Subject | 0.16 | | | |
| Marginal R^2 / Conditional R^2 | 0.305 / 0.668 | | | |
| AIC | 54.205 | | | |

3.2 Defining behavioural stress reactivity

The time it took for individuals to approach all three novel containers was significantly longer than for the control container (Control: mean=3.23 \pm 0.36 seconds; N1: mean=377.62 \pm 39.75 seconds, $p < 0.001$; N2: mean=222.38 \pm 91.22 seconds, $p < 0.01$; N3: mean=211.08 \pm 94.52, $p = 0.01$). The time taken to retrieve the mealworm was highly variable among individuals, but very consistent within individuals (Fig. 9; Fig. 10). Overall, individuals took slightly longer to retrieve the mealworm in the first and second trials (N1 and N2) than for the final (N3) (Fig. 9), perhaps indicating learning, consequently N3 was excluded from the stress reactivity PCA (Fig. 11).

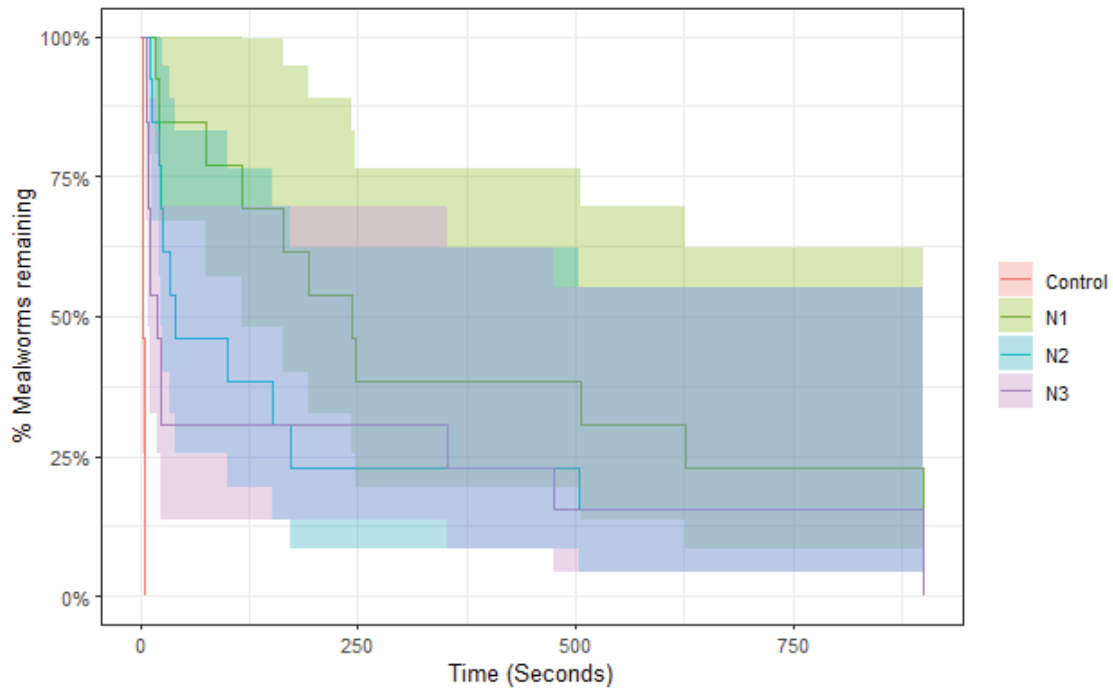


Figure 9. Kaplan Meier survival model displaying the rate that birds took a mealworm from three novel containers. Coloured lines indicate the percentage of mealworms taken by the birds from each container type over the given time frame: N1 (green), N2 (blue) and N3 (purple), are the three different novel container types (Fig. 1), and the control was a regular terracotta food container (orange). Coloured bands indicate 95% confidence intervals.

Object neophobia was positively correlated with the handling stress behavioural scores (Fig. 10; Conditional $R^2 = 0.86$; $P = 0.015$; supplementary table 1); individuals that showed less caution in approaching a novel container also tended to be more stress reactive during handling.

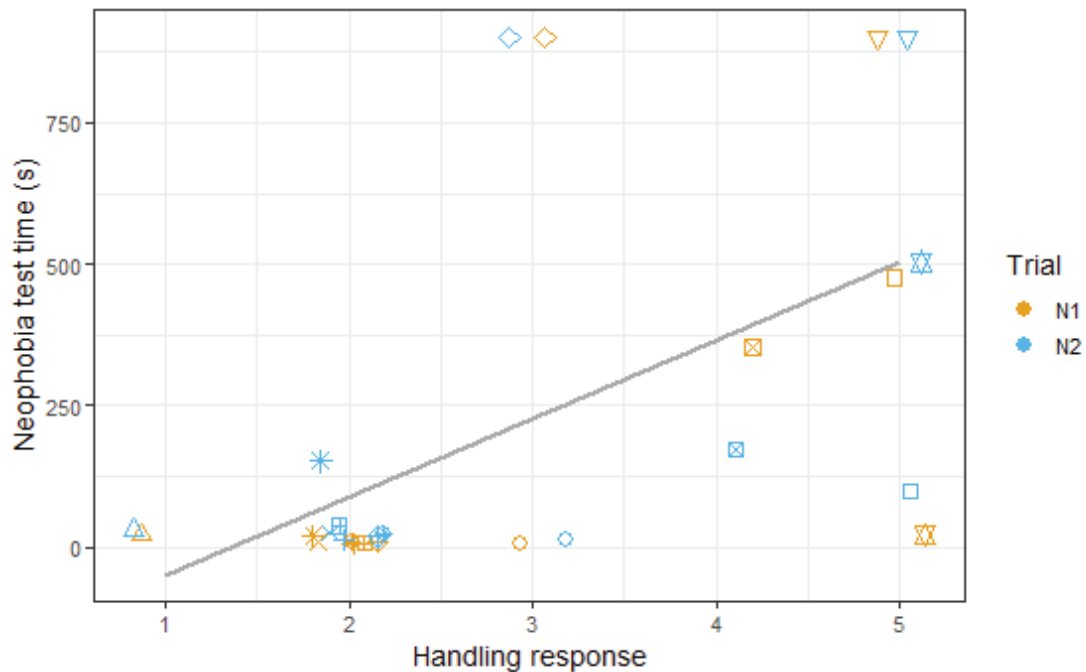


Figure 10. Linear regression showing neophobia trials 1 and 2 times, against handling response given by their behavioural stress during handling rating. The neophobia trial number is indicated by colour, N1 in orange and N2 in blue (Fig. 1). Different subjects are indicated by different point shapes (N=13). Linear regression line is indicated in grey, where conditional $R^2 = 0.86$ and slope = 137.8.

Principal component one (PC1) accounted for the variation among individuals very well (59.9%, Fig. 11). Individuals with a low PC1 had a low handling stress rating and fast retrieval times. Males tended to have higher stress reactivities than females (mean stress reactivity score females = -0.57, males = 0.49) although this difference was not significant ($t = -1.22$, $p = 0.25$, 95% CI = -2.98, 0.85).

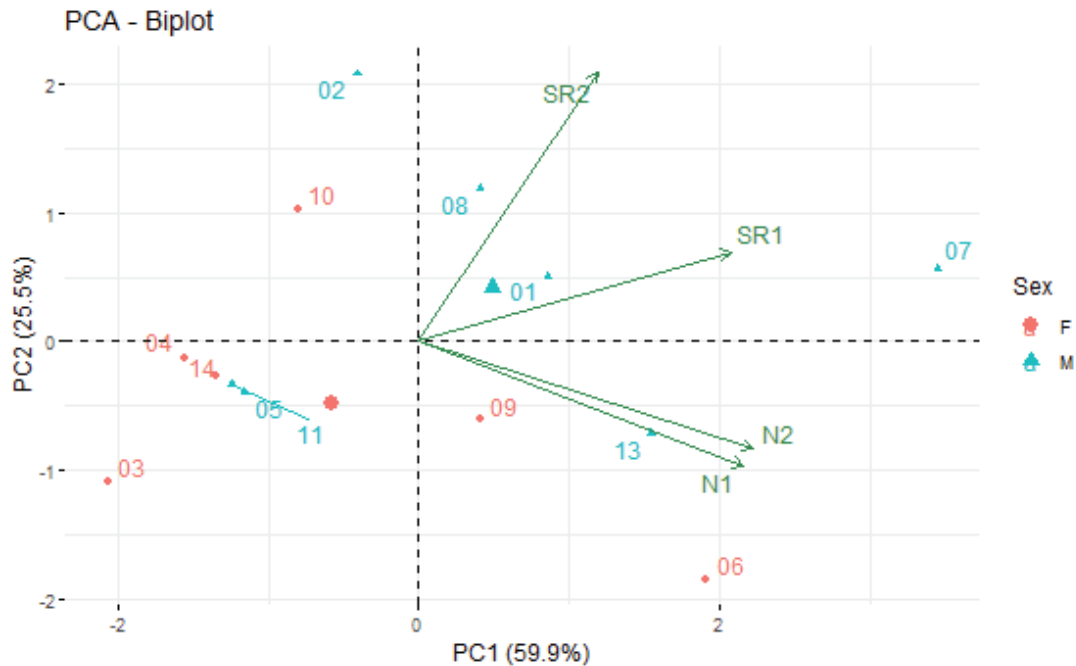


Figure 11. Biplot of a principal component analysis that included the number of seconds to retrieve a mealworm from a novel container (N1 and N2), and ratings (1-5) given by handling stress measurements taken on two different days (SR1 and SR2) shown in green. The point and label indicate each subject ID, with sex indicated in red and blue (female and male). The large red circle, and large blue triangle indicate the average placement of the females and males respectively. Variables were scaled in the PCA. Principal component 1 (PC1) accounts for 59.9%, and principal component 2 accounts for 25.5% of the variance. See supplementary table 2 for variable loadings and eigenvalues. Variation between individuals in PC1 was used as a measurement of stress reactivity.

3.3 How does stress reactivity score relate to measures of stress

The relationship between stress reactivity score and eye temperature depended on sex and whether birds have experimentally elevated CORT levels (Fig. 12; Table 7). In males, individuals with low-stress reactivity scores had higher eye temperatures during baseline and followed a similar pattern at elevated CORT (Fig 12; Table 7). In females, individuals with low-stress reactivity scores had lower eye temperatures during baseline, but showed the opposite pattern when elevated, individuals with low-stress reactivity scores had higher eye temperatures. Eye temperatures were also higher when CORT was elevated experimentally (0.35°C increase first five minutes following CORT ingestion). Females had higher

temperatures than males (0.48°C difference during the first five minutes following CORT ingestion).

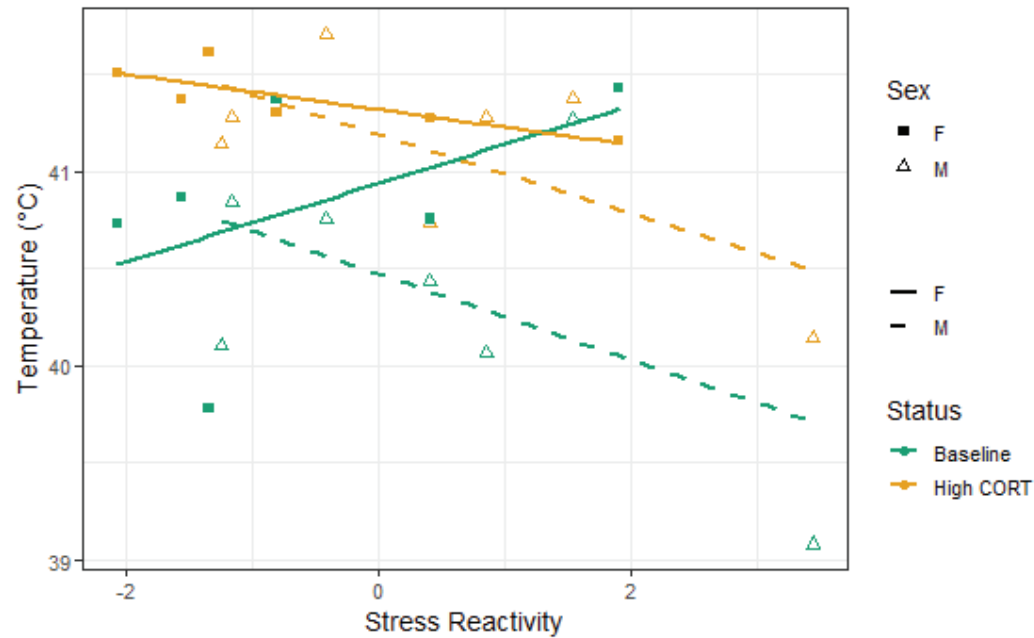


Figure 12. Linear regression models showing stress reactivity principal component one (PC1) correlation with mean temperature, during the first 5 minutes following CORT ingestion (elevated) compared to the 5 minutes preceding CORT ingestion (baseline) and by sex. CORT treatment is indicated by colour. Points and linear regression line of eye temperature when CORT treatment is elevated (high CORT) are indicated in yellow. Points and linear regression of eye temperature when CORT is at baseline is indicated in green (see table 7). Sex is indicated by point shape and line type, (square and solid line are females, triangle and dotted line are male).

Table 7. Summary of a linear mixed model (LMM) results showing the effects of stress reactivity, sex(m/f) and CORT treatment (baseline or elevated) on individual bird eye temperature. Baseline CORT treatment measurements were taken in the five minutes before CORT was ingested, and elevated CORT treatment measurements were taken in the five minutes after the birds ingested a mealworm injected with 20ug of CORT). The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold, marginal estimates where $P < 0.01$ are indicated in italics.

| <i>Predictors</i> | Temperature | | | |
|----------------------------------------------------|--------------------|---------------|------------------|------------------|
| | <i>Estimates</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept) | 40.93 | 40.58 – 41.29 | 224.95 | <0.001 |
| Treatment [Elevated] | 0.35 | 0.08 – 0.63 | 2.49 | 0.013 |
| Sex [M] | -0.48 | -0.96 – -0.00 | -1.97 | 0.049 |
| Stress reactivity | 0.18 | -0.06 – 0.43 | 1.47 | 0.141 |
| Treatment [Elevated] * Sex [M] | 0.36 | -0.01 – 0.74 | 1.90 | <i>0.058</i> |
| Treatment [Elevated] * Stress reactivity | -0.27 | -0.46 – -0.08 | -2.75 | 0.006 |
| Sex [M] * Stress reactivity | -0.42 | -0.73 – -0.11 | -2.64 | 0.008 |
| Treatment [Elevated] * Sex [M] * Stress reactivity | 0.29 | 0.05 – 0.54 | 2.34 | 0.019 |
| Random Effects | | | | |
| Residual effect | 0.28 | | | |
| Individual effect | 0.11 | | | |
| Observations | 143 | | | |
| Marginal R^2 / Conditional R^2 | 0.355 / 0.539 | | | |
| AIC | 278.695 | | | |

The relationship between stress reactivity score and faecal CORT concentration was marginally dependant on sex and whether birds have experimentally elevated CORT levels (Fig. 13; Table 8). Faecal CORT was marginally higher in individuals with higher stress reactivity overall (Fig. 13; Table 8). Males had significantly lower faecal CORT concentrations overall (Fig. 13; Table 8). As with eye temperature and reported previously in

section 3.1 ‘Using thermal imagery to measure stress’, faecal CORT levels were higher when experimentally elevated (Table 8).

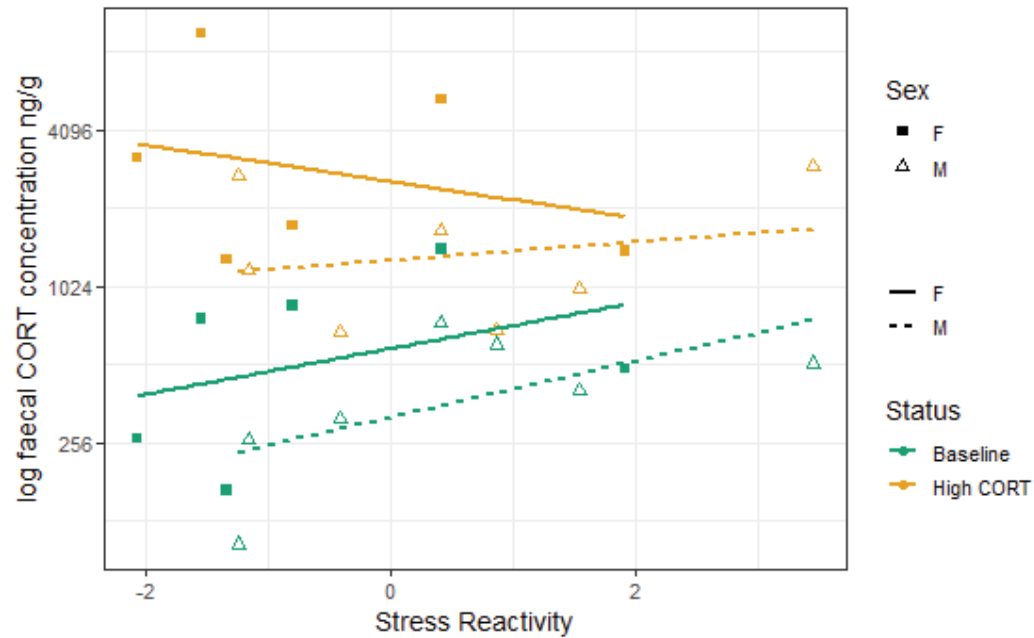


Figure 13. Linear regression models show the relationship between stress reactivity score principal component one (PC1) and faecal corticosterone according to treatment and sex. Treatment is indicated by colour (baseline, indicated in green, and elevated stress (ingestion of a mealworm injected with 20ug of crystalline CORT), indicated in yellow). Sex is indicated by point shape and line type, (square and solid line are females, triangle and dotted line are male).

Table 8. Summary of a linear mixed model (LMM) results showing how logged faecal CORT concentration (ng/g) is affected by stress reactivity, sex (m/f) and CORT treatment (baseline and elevated [ingestion of mealworm injected with 20ug of CORT dissolved in DMSO]). The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold.

| <i>Predictors</i> | Log faecal CORT concentration ng/g | | | |
|------------------------------------------------------|-------------------------------------------|---------------|------------------|------------------|
| | <i>Estimates</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept) | 6.43 | 5.92 – 6.93 | 24.88 | <0.001 |
| Treatment [High CORT] | 1.48 | 1.04 – 1.93 | 6.48 | <0.001 |
| Stress reactivity | 0.24 | -0.01 – 0.49 | 1.86 | 0.063 |
| Sex [M] | -0.67 | -1.31 – -0.04 | -2.08 | 0.038 |
| Treatment [High CORT] * Stress reactivity | -0.26 | -0.55 – 0.03 | -1.76 | 0.078 |
| Random Effects | | | | |
| Residual effect | 0.34 | | | |
| Individual effect | 0.13 | | | |
| Marginal R ² / Conditional R ² | 0.605 / 0.714 | | | |
| AIC | 70.410 | | | |

3.4 How does stress reactivity relate to behavioural responses to stress?

Neither preening or drinking was related to treatment or stress reactivity. For newspaper rustles, there was only a detectable effect of treatment when extreme individuals were excluded, there was a marginal trend for fewer newspaper rustles when CORT was elevated (Fig. 14b; Table 9; Table 10)

However, position moves were positively related to stress reactivity score and were overall higher when CORT was elevated when including all values (Fig. 14a; Table 9). However, this relationship was driven by the extreme measurements; this relationship was not seen when extreme values were excluded (Table 10). However, though there was no relationship between reactivity score and position moves after excluding extremes, the number of position

moves was lower when CORT was elevated (Table 10). The number of position ‘moves’ for males was lower still, when CORT was elevated.

The number of feather ruffles was unrelated to individual reactivity score but did decrease when CORT was elevated, and extreme values did not qualitatively alter this relationship (Fig. 14a, Table 9, Table 10). No birds were observed panting through the duration of these observations.

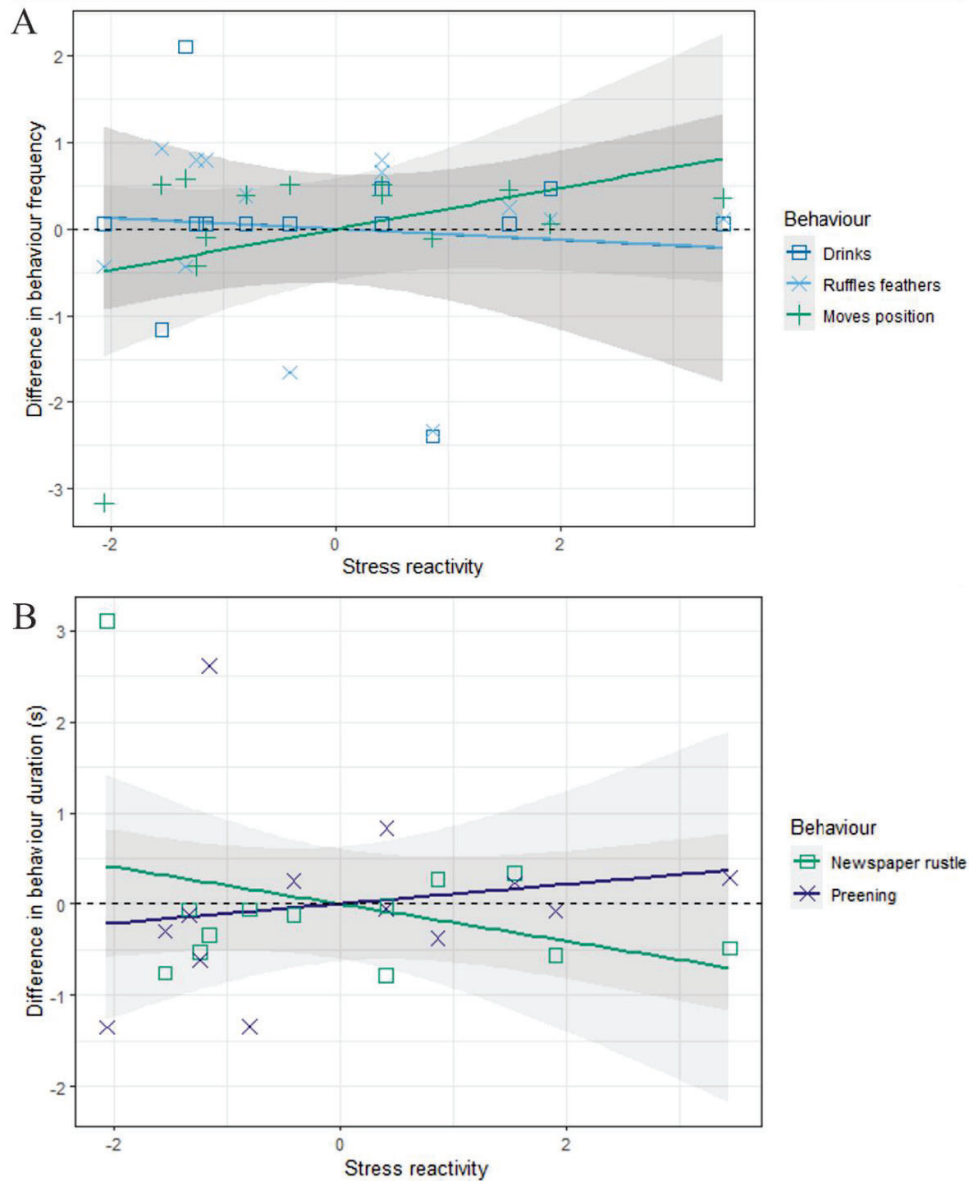


Figure 14. Scatterplots show a scaled change in the activity of certain behaviours 15 minutes before and after ingesting mealworm injected with 20ug of CORT against stress reactivity. Colour indicates behaviour types, and coloured lines indicate linear regression of difference in activity of that behaviour, with standard error indicated in grey colour. A) Difference in the frequency of behaviours; ‘drink’ (dark blue squares), ‘ruffles feathers’ (light blue, X) and ‘moves position’ (green, +). B) Difference in the duration of behaviours ‘newspaper rustle’ (green squares), ‘preening’ (dark blue X). The dotted black line indicates 0 where behaviour frequency or duration decreased or increased after ingesting the CORT mealworm.

Table 9. Summary of generalised linear mixed models (GLMMs) and linear mixed models (LMMs) showing the relationships between behaviours, CORT treatment and stress reactivity. CORT treatments included: baseline, where CORT was not altered and elevated, birds ingested a mealworm injected with 20ug of CORT. Behaviours measured by frequency were tested using Poisson distribution and measured by duration using Gaussian. Values where text is dark yellow are reported estimates and P values prior to exclusion from the final model. The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold, marginal estimates where $P < 0.01$ are indicated in italics. These models include extreme values (outside of three times the standard deviation), (G)LMMs without extreme values are presented in Table 10.

| | Dependent variable including extreme values: | | | | |
|---------------------------------------|----------------------------------------------|---------------------|------------------|--------------------------|--------------------------|
| | Duration (LMM) | | Frequency (GLMM) | | |
| | Newspaper rustle | Preening | Drinking | Moves position | Ruffle feathers |
| Intercept | 47.897 (28.851)* | 105.563 (29.654)*** | -1.557 (1.012) | 2.949 (0.229)*** | 2.118 (0.132)*** |
| Stress reactivity | p = 0.097 | p = 0.0004 | p = 0.125 | p < 0.0001 | p < 0.0001 |
| | -3.094 (18.636) | -24.896 (19.155) | -0.235 (0.421) | -0.369 (0.151)** | 0.050 (0.086) |
| Treatment(Elevated) | p = 0.869 | p = 0.194 | p = 0.576 | p = 0.015 | p = 0.561 |
| | 8.498 (36.529) | -40.040 (39.641) | 0.695 (0.856) | -1.285 (0.119)*** | -0.801 (0.168)*** |
| Sex(M) | p = 0.817 | p = 0.313 | p = 0.417 | p < 0.0001 | p = 0.00001 |
| | 4.303 (63.993) | -11.235 (63.524) | 1.015 (1.187) | 0.046 (0.482) | 0.401 (0.261) |
| Stress reactivity*Treatment(Elevated) | p = 0.947 | p = 0.860 | p = 0.393 | p = 0.924 | p = 0.125 |
| | -27.091 (23.596) | 14.800 (25.607) | 0.002 (0.465) | 0.412 (0.077)*** | -0.066 (0.117) |
| Sex(M)*Treatment(Elevated) | p = 0.251 | p = 0.564 | p = 0.997 | p < 0.0001 | p = 0.572 |
| | -44.284 (80.613) | 97.260 (83.295) | -1.677 (1.174) | 0.042 (0.251) | -0.267 (0.356) |
| | p = 0.583 | p = 0.243 | p = 0.154 | p = 0.867 | p = 0.454 |
| Residual effect | 8673 | 10214 | | | |
| Individual effect | 2147 | 1217 | 1.694 | 0.6123 | 0.1018 |
| Akaike Inf. Crit. | 290.362 | 291.886 | 79.057 | 282.682 | 159.791 |
| Note: | * p<0.1; ** p<0.05; *** p<0.01 | | | | |

Table 10. Summary of generalised linear mixed models (GLMMs) and linear mixed models (LMMs) showing the relationships between behaviours, CORT treatment and stress reactivity. CORT treatments included: baseline, where CORT was not altered, elevated, and birds ingested a mealworm injected with 20ug of CORT. Behaviours measured by frequency were tested using Poisson distribution and measured by duration using Gaussian. Values where text is dark yellow are reported estimates and P values prior to exclusion from the final model. The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold, marginal estimates where $P < 0.01$ are indicated in italics. These models exclude extreme values (outside of three times the standard deviation); (G)LMMs that include extreme values are presented in Table 9.

| | <i>Dependent variable without extreme values:</i> | | | | |
|---------------------------------------|---------------------------------------------------|--------------------------------|-------------------------|-------------------------------|-------------------------------|
| | <i>Duration (LMM)</i> | | <i>Frequency (GLMM)</i> | | |
| | Newspaper rustle | Preening | Drinking | Moves position | Ruffles feathers |
| Intercept | 43.123 (10.056) ^{***} | 98.596 (31.538) ^{***} | -0.673 (0.602) | 2.412 (0.295) ^{***} | 2.115 (0.139) ^{***} |
| Stress reactivity | p = 0.00002 | p = 0.002 | p = 0.264 | p < 0.0001 | p < 0.0001 |
| | 1.024 (6.719) | -18.888 (21.071) | -0.189 (0.366) | -0.128 (0.132) | 0.052 (0.094) |
| Treatment(Elevated) | p = 0.879 | p = 0.371 | p = 0.606 | p = 0.332 | p = 0.579 |
| | -26.591 (14.222) [*] | -24.619 (40.385) | -0.326 (0.484) | -0.429 (0.199) ^{**} | -0.711 (0.170) ^{***} |
| Sex(M) | p = 0.062 | p = 0.543 | p = 0.501 | p = 0.031 | p = 0.00004 |
| | 11.550 (21.541) | -1.445 (66.814) | 0.773 (1.093) | 0.541 (0.387) | 0.434 (0.277) |
| Stress reactivity*Treatment(Elevated) | p = 0.592 | p = 0.983 | p = 0.480 | p = 0.162 | p = 0.118 |
| | 3.170 (9.502) | 1.500 (26.982) | -0.404 (0.401) | -0.002 (0.099) | -0.164 (0.128) |
| Sex(M)*Treatment(Elevated) | p = 0.739 | p = 0.956 | p = 0.315 | p = 0.986 | p = 0.199 |
| | 5.578 (30.464) | 78.834 (84.709) | -1.601 (1.132) | -0.775 (0.257) ^{***} | -0.449 (0.357) |
| | p = 0.855 | p = 0.353 | p = 0.158 | p = 0.003 | p = 0.209 |
| Residual effect | 0 | 9656 | | | |
| Individual effect | 1197 | 2121 | 1.335 | 0.3439 | 0.1043 |
| Akaike Inf. Crit. | 222.044 | 267.433 | 74.885 | 214.125 | 147.002 |
| <i>Note:</i> | | | | | |
| *p<0.1; **p<0.05; *** p<0.01 | | | | | |

3.5 The effect of stress reactivity, CORT treatment and sex on singing

The LM testing for differences in singing activity among individuals with different stress reactivities throughout two days of baseline stress showed considerable variation among individuals (mean = 364.36 seconds, CI = 228.20 – 500.53). Variation in total singing time was significantly affected by day (Table 11). There is moderate evidence that individuals with higher stress reactivities sang more than those with lower scores (Table 11; Fig. 15). Male birds also sang marginally more than females (means: female = 384.6 seconds, male = 700.1 seconds, Fig. 15, Table 11).

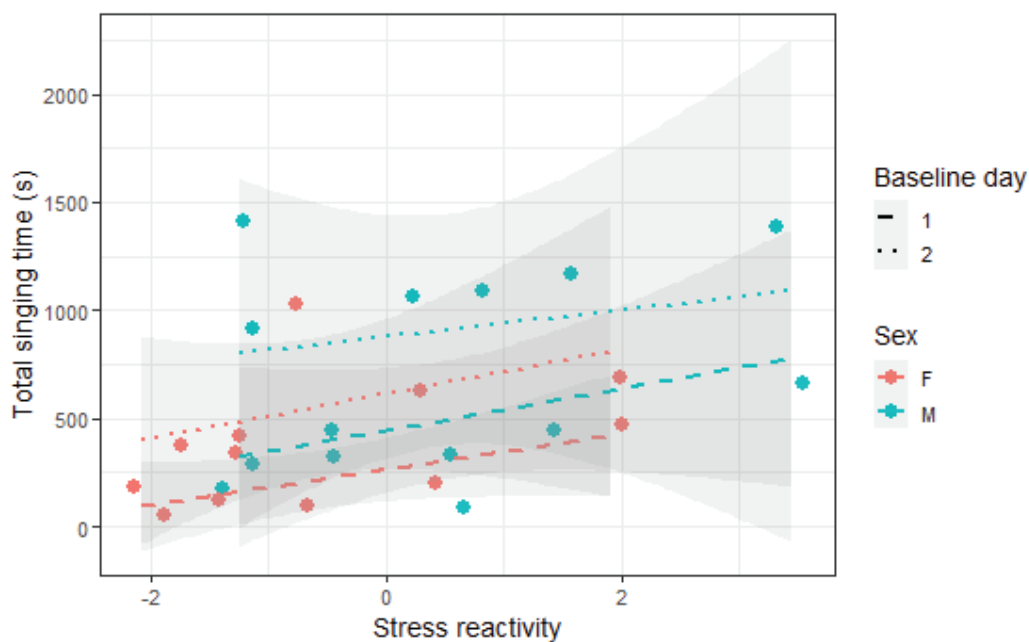


Figure 15. Scatterplot presenting two days total singing time while CORT was not experimentally manipulated, but instead predicted by individual stress reactivity scores. Coloured regression lines indicate day (day 1 = dashed line, day 2 = dotted line), and sex (female = red, male = blue). Grey bands indicate confidence intervals of each linear regression.

The LMM testing for effects of CORT manipulation on singing activity showed the birds' total singing activity significantly increased after CORT ingestion (Table 12; Fig. 16). Individuals that were more stress reactive also sang significantly more than less stress reactive individuals in both treatments (Table 12; Fig. 16).

Table 11. Summary of linear model (LM) results show stress reactivity and sex effects on singing activity throughout two baseline days (during the first five minutes of every hour from 0600h-1800h). The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold, marginal estimates where $p < 0.8$ are indicated in italics.

| Total singing activity throughout two non-experimental days (seconds) | | | | |
|-----------------------------------------------------------------------|------------------|-----------------|------------------|--------------|
| <i>Predictors</i> | <i>Estimates</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept) | 243.01 | 15.73 – 470.29 | 2.10 | 0.036 |
| Stress reactivity | 84.56 | -1.19 – 170.32 | 1.93 | <i>0.053</i> |
| Sex [M] | 225.37 | -40.92 – 491.66 | 1.66 | <i>0.097</i> |
| Date [Day 2] | 380.25 | 130.87 – 629.63 | 2.99 | 0.003 |
| Random Effects | | | | |
| Residual effect | 105231.87 | | | |
| Individual effect | 0.00 | | | |
| Marginal R^2 / Conditional R^2 | 0.429 / NA | | | |
| AIC | 339.842 | | | |

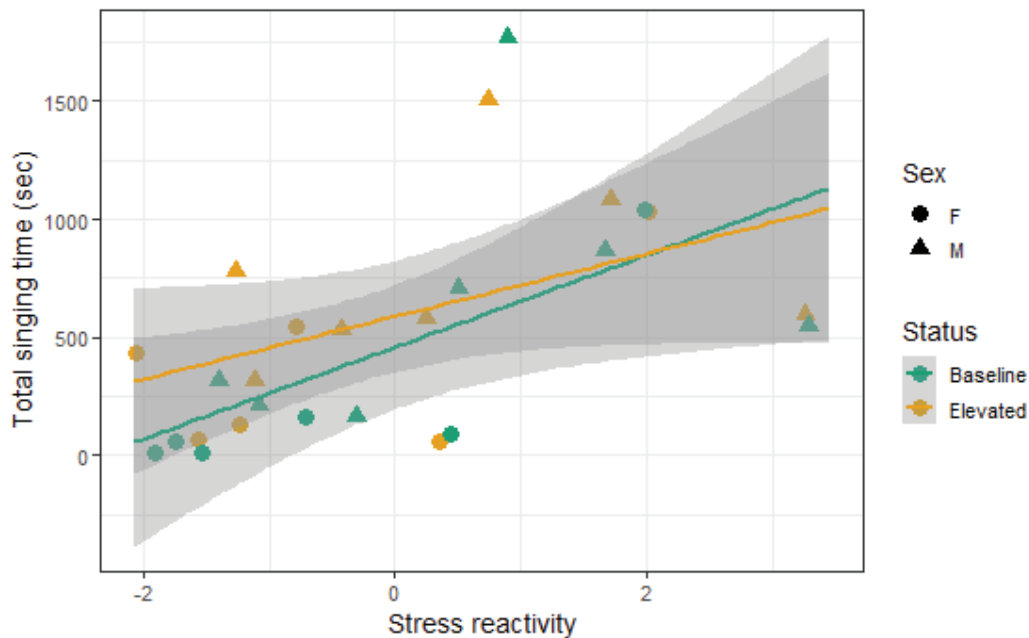


Figure 16. Scatterplot presenting the relationship between stress reactivity and total time spent singing where CORT was experimentally manipulated. Coloured regression lines indicate CORT treatment: baseline (green) where CORT was not altered, and elevated (yellow) after ingestion of mealworm injected with 20ug of CORT. Sex is indicated by symbol shape (females = circles and males = triangles). Grey bands indicate confidence intervals of each linear regression.

Table 12. Summary of a linear mixed model (LMM) showing total time spent singing as predicted by stress reactivity and CORT treatment (baseline being where CORT was not altered, elevated treatment indicates ingestion of mealworm injected with 20ug of CORT). Significant values where $p < 0.05$ are indicated in bold. The variance attributed to the random effects of Subject ID and the residual are presented for the model.

| <i>Predictors</i> | Total singing time during experiment (seconds) | | | |
|---------------------------------------|-------------------------------------------------------|-----------------|------------------|------------------|
| | <i>Estimates</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept) | 459.05 | 236.56 – 681.54 | 4.04 | <0.001 |
| Stress reactivity | 195.09 | 51.37 – 338.81 | 2.66 | 0.008 |
| Treatment(Elevated) | 129.67 | 4.20 – 255.13 | 2.03 | 0.043 |
| Stress reactivity*Treatment(Elevated) | -62.48 | -143.53 – 18.56 | -1.51 | 0.131 |
| Random Effects | | | | |
| Residual effect | 26635.82 | | | |
| Individual effect | 140883.04 | | | |
| Marginal R^2 / Conditional R^2 | 0.306 / 0.890 | | | |
| AIC | 337.562 | | | |

The LMMs testing for relationships between sex, stress reactivity, and song complexity measures revealed several important interactions. The relationships between stress reactivity, syllable score, maximum frequency and bandwidth depended on sex and whether birds had experimentally elevated CORT (Fig. 17; Fig. 18).

During the hour and a half following CORT ingestion, the birds' song complexity significantly shifted: syllable switches, syllable score, maximum frequency and spectral flux were higher, and mean bandwidth was reduced relative to songs during baseline (Fig. 17; Fig. 18). Further, more stress reactive individuals had significantly higher syllable diversity and bandwidth, fewer switches and lower maximum frequency than less stress reactive individuals. After CORT ingestion, males had significantly fewer syllable switches, a lower syllable score and maximum frequency, but higher mean bandwidth than females.

Overall, more stress reactive individuals had significantly more syllable switches, higher syllable score and maximum frequency, and lower bandwidth than less stress reactive individuals (Fig. 17; Fig. 18). More stress reactive females had a significantly higher syllable

score, maximum frequency, more syllable switches and marginally higher spectral flux than males (Fig. 17; Fig. 18). However, overall, males had significantly more syllable switches, higher syllable scores, maximum frequency and flux, and lower bandwidth than females. Syllable rate did not show any relationship with any of the fixed effects (Fig. 17; Fig. 18). For more details on song complexity interactions, see supplementary tables 3 and 4.

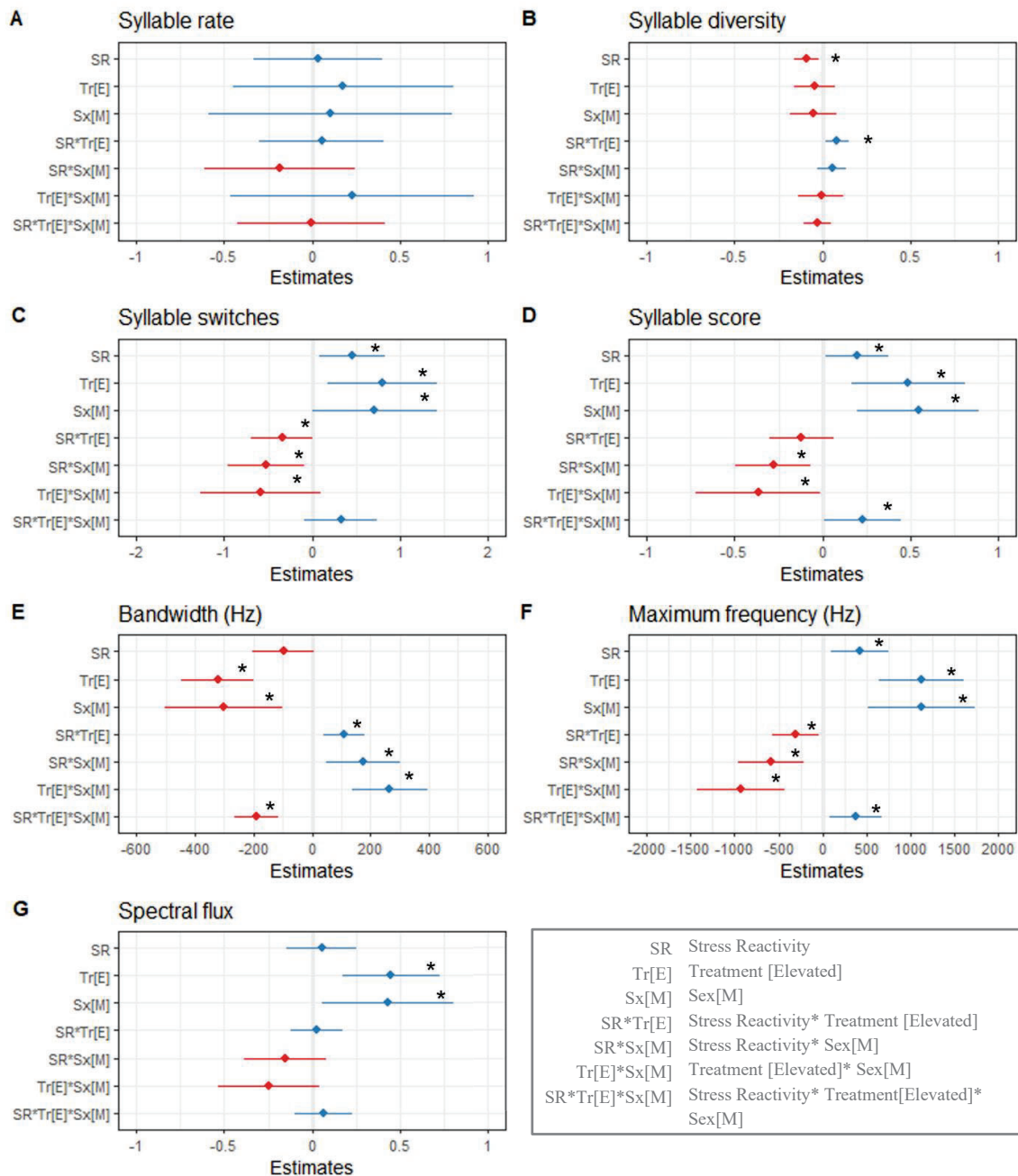


Figure 17. Linear mixed model results presenting the predicted effects between mean parameters of song complexity and individual stress reactivity, incorporating the interactions of CORT treatment (baseline/elevated) and sex (m/f). Estimates are indicated by centre dot of each line. The upper and lower edges of each line indicate 95% confidence intervals, significant estimates are indicated '*'. Effect direction is indicated by colour, where positive effects are blue, and negative effects are red. See Table 1 for details on parameters of song complexity. LMMs of each relationship are presented in Supplementary Tables 3 and 4.

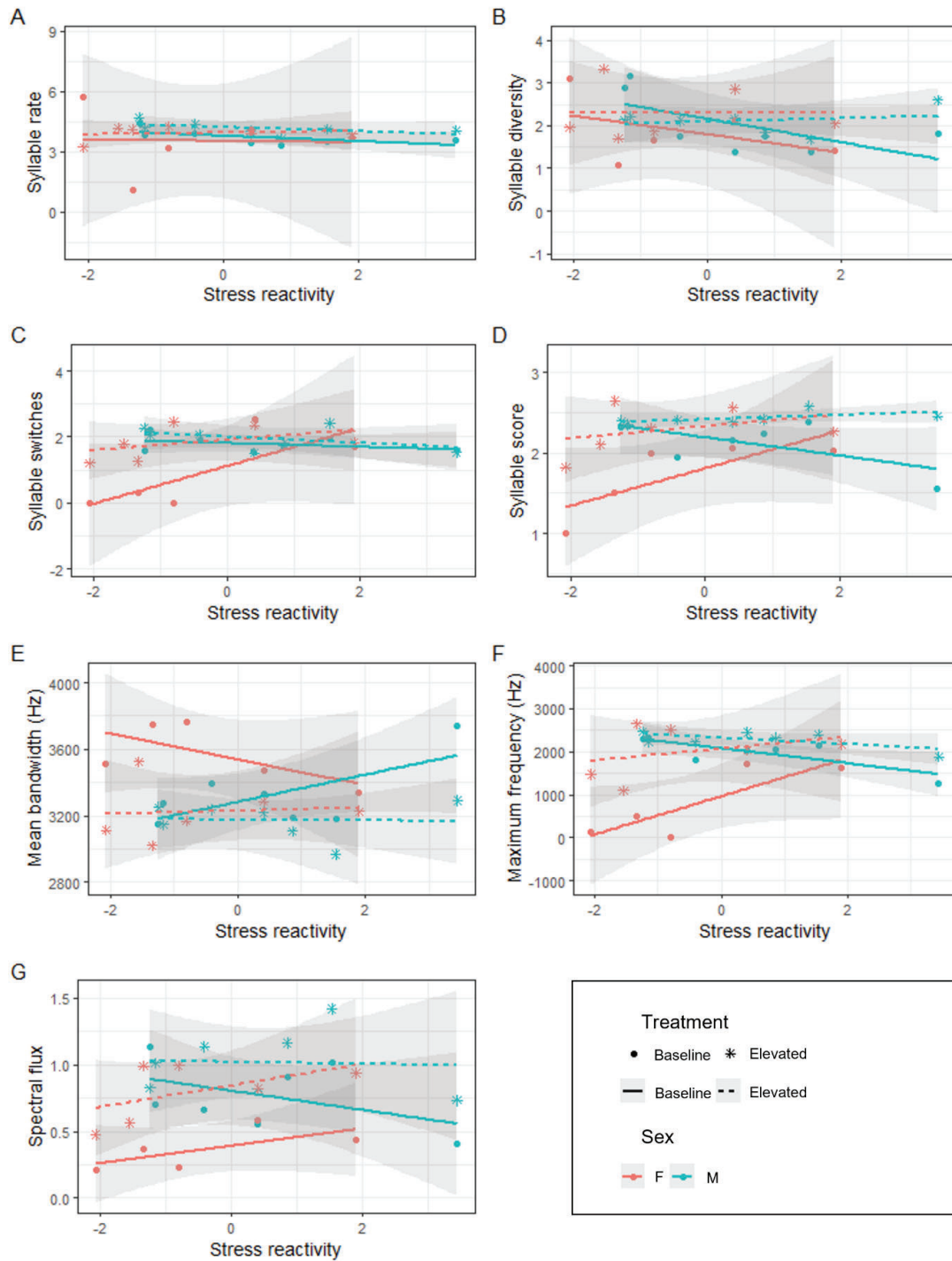


Figure 18. Scatterplots presenting the relationship between mean parameters of song complexity and individual stress reactivity, incorporating the interactions of CORT treatment (baseline/elevated) and sex (m/f). Sex is indicated by colour, where females are red and males are blue. CORT treatment is indicated by line type, and point type, where baseline treatment is the solid line and solid circle, and elevated treatment is the dashed line and asterisk point. Confidence intervals for each line are indicated in shaded grey. See Table 1 for details on parameters of song complexity. LMMs of each relationship are presented in Supplementary Tables 3 and 4.

The LMMs that tested which stress measure best explains parameters of song complexity found different relationships for baseline and elevated treatments (Table 13; Table 14). At baseline, none of the predictive variables were effective for bandwidth or spectral flux. The best model for maximum frequencies was a *sex x reactivity* interaction (Table 13). Syllable switches and total time singing were best predicted by stress reactivity, though the relationship for syllable switches differed according to sex. Syllable diversity was related to all three stress parameters – higher faecal CORT, eye temperature and reactivity scores all predicted lower syllable diversity. Syllable score was best predicted by temperature, but was also predicted by stress reactivity, which differed slightly according to sex. Syllable rate was also best predicted by temperature, which differed according to sex, but was also predicted by stress reactivity, and *faecal CORT x sex*.

During elevated treatment, stress reactivity was the best at predicting syllable score, and partially predicted spectral flux. Syllable diversity was best predicted by eye temperature, and syllable switches was best predicted by *eye temperature x sex* (Fig. 19). Faecal CORT was the best predictor of song parameters during elevated CORT; as faecal CORT best predicted bandwidth, spectral flux, maximum frequency and total time singing. Faecal CORT also explained syllable diversity. Further, *faecal CORT x sex* best predicted syllable rate, and predicted maximum frequency, syllable rate and syllable diversity (Table 14).

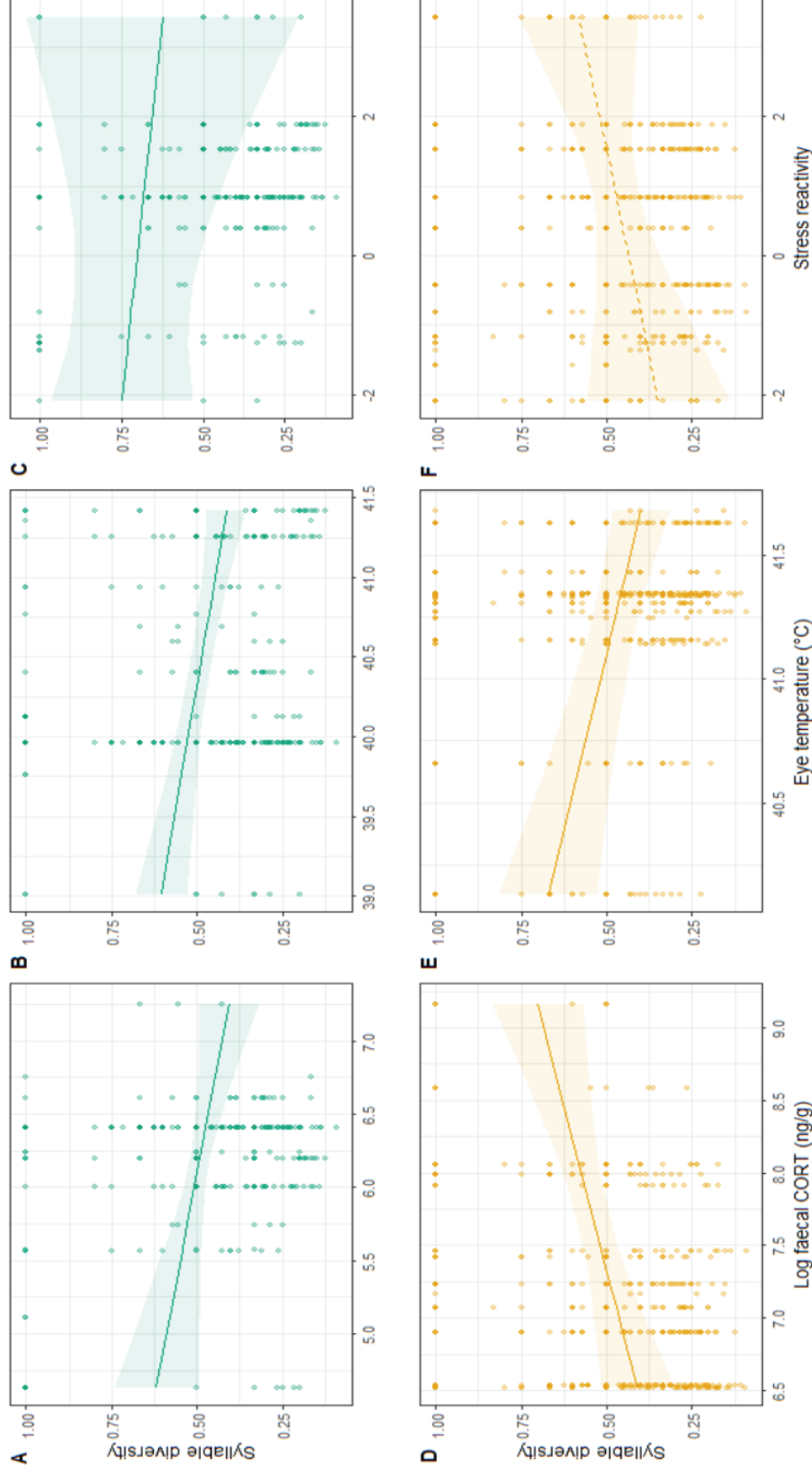


Figure 19. Example of how well measurements of stress predict parameters of song complexity; linear mixed model (LMM) results for the effects of log faecal CORT, eye temperature and stress reactivity on song syllable diversity, at baseline and elevated CORT. Colour indicates CORT treatment, where the top row (A,B,C) in green, is baseline CORT. The bottom row (D,E,F) is elevated CORT. Confidence intervals for each prediction are highlighted in yellow or green. The dots indicate individual songs. Complete LMMs output of each parameter of song complexity are presented in tables G3 and G4.

Table 13. Summary of linear mixed models (LMMs) and a linear regression of song complexity measures when CORT was at baseline. Values where text is dark yellow are reported estimates and P values prior to exclusion from the final model. Significant values where $p < 0.05$ are indicated in bold, and $p < 0.1$ are indicated in italics. The variance attributed to the random effects of Subject ID and the residual are presented for the model.

| <i>Song complexity measures when stress is at baseline: Dependent variable:</i> | | | | | | | | |
|----------------------------------------------------------------------------------------|------------------------------|-----------------------|----------------------------|-----------------------|-------------------------|-----------------------|------------------------|-----------------------------|
| | Bandwidth (Hz) | Spectral flux | Maximum frequency (Hz) | Syllable switches | Syllable rate | Syllable score | Syllable diversity | Total time singing (sec) |
| | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>Linear regression</i> |
| Intercept | 7,731.54 (3,672.04)** | 0.41 (0.14)*** | 982.48 (244.85)*** | 0.99 (0.28)*** | -63.23 (29.42)** | -5.99 (4.43) | 4.22 (1.07)*** | 459.05 (119.68)*** |
| Sex(M) | p = 0.036 | p = 0.005 | p = 0.0001 | p = 0.0004 | p = 0.032 | p = 0.177 | p = 0.0001 | p = 0.003 |
| | -296.15 (115.04)** | 0.37 (0.17)** | 1,172.54 (259)*** | 0.77 (0.3)*** | 70.98 (30.18)** | 0.66 (0.19)*** | -5.90 (6.19) | -3,366.93 (2,704.61) |
| | p = 0.011 | p = 0.027 | p = 0.00001 | p = 0.010 | p = 0.019 | p = 0.001 | p = 0.341 | p = 0.254 |
| Stress reactivity | -62.014 (62.19) | 0.02 (0.07) | 352.24 (138.31)** | 0.41 (0.15)*** | -0.42 (0.24)* | 0.14 (0.10) | -0.03 (0.02)* | 195.09 (77.31)** |
| | p = 0.319 | p = 0.766 | p = 0.011 | p = 0.008 | p = 0.084 | p = 0.145 | p = 0.066 | p = 0.029 |
| Temperature (°C) | -102.83 (89.63) | 0.03 (0.14) | -965.35 (698.52) | -0.95 (0.77) | 1.58 (0.73)** | 0.19 (0.11)* | -0.08 (0.02)*** | 352.81 (515.07) |
| | p = 0.252 | p = 0.858 | p = 0.167 | p = 0.216 | p = 0.031 | p = 0.082 | p = 0.002 | p = 0.524 |
| Log faecal CORT | 24.03 (97.85) | 0.12 (0.22) | 293.79 (359.7) | 0.73 (0.50) | 0.35 (0.47) | -0.05 (0.21) | -0.08 (0.04)** | -150.53 (273.01) |
| | p = 0.807 | p = 0.582 | p = 0.415 | p = 0.147 | p = 0.458 | p = 0.797 | p = 0.035 | p = 0.599 |
| Sex(M)*Stress reactivity | 119.54 (79.06) | -0.061 (0.168) | -493.93 (151.39)*** | -0.43 (0.18)** | 0.40 (0.26) | -0.20 (0.12)* | -0.00 (0.05) | -253.09 (203.7) |
| | p = 0.131 | p = 0.716 | p = 0.002 | p = 0.017 | p = 0.129 | p = 0.086 | p = 0.964 | p = 0.255 |
| Sex(M)*Temperature (°C) | -122.84 (291.70) | -0.34 (0.25) | 1,073.51 (719.02) | 1.17 (0.78) | -1.60 (0.75)** | 0.36 (0.48) | 0.15 (0.15) | 607.16 (448.32) |
| | p = 0.674 | p = 0.179 | p = 0.136 | p = 0.135 | p = 0.032 | p = 0.458 | p = 0.324 | p = 0.218 |

| | | | | | | | | |
|------------------------------------------|--------------------------------|---------------------------|-----------------------------------|---------------------------|----------------------------|---------------------------|---------------------------|------------------------------|
| Sex(M)*Log faecal CORT | -26.35 (224.94) p = 0.907 | -0.36 (0.32) p = 0.272 | 1,142.85 (704.64) p = 0.105 | -0.74 (0.55) p = 0.179 | -0.91 (0.53)* p = 0.084 | -0.26 (0.35) p = 0.451 | -0.06 (0.11) p = 0.552 | 757.20 (554.54) p = 0.231 |
| Residual effect | 219174 | 0.72840 | 3359598 | 1.672 | 1.422 | 0.545379 | 0.06318 | NA |
| Individual effect | 19251 | 0.04529 | 15759 | 0 | 0 | 0.008704 | 0 | NA |
| Observations | 2,640 | 2,640 | 2,640 | 282 | 264 | 282 | 282 | 13 |
| R ² / Adjusted R ² | | | | | | | | 0.367/0.309 |
| Akaike Inf. Crit. | 39,940.320 | 6,687.713 | 47,134.060 | 962.677 | 865.448 | 656.585 | 52.932 | |
| Note: | * p<0.1; ** p<0.05; *** p<0.01 | | | | | | | |

Table 14. Summary of linear mixed models (LMMs) and linear regression of song complexity measures when CORT was elevated (following ingestion of mealworm injected with 20ug of CORT). Values indicated in dark yellow are reported estimates and P values prior to exclusion from the final model. Significant values where $p < 0.05$ are indicated in bold, and $p < 0.1$ are indicated in italics. The variance attributed to the random effects of Subject ID and the residual are presented for the model.

| Song complexity measures when CORT is at elevated : Dependent variable: | | | | | | | | |
|--------------------------------------------------------------------------------|--------------------------------------|-----------------------------------------|---------------------------------------------------------------|-------------------------------------------------|-----------------------------------|--------------------------------------------------------|------------------------------------------------------|-------------------------------------------------|
| | Bandwidth (Hz) | Spectral flux | Maximum frequency (Hz) | Syllable switches | Syllable rate | Syllable score | Syllable diversity | Total time singing (sec) |
| | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>LMM</i> | <i>Linear regression</i> |
| Intercept | 11,142.85 (8,634.61) | -8.95 (8.04) | 6,676.31 (1,522.26)*** | 137.54 (84.65) | 42.09 (48.95) | 3.07 (0.52)*** | 6.95 (2.43)*** | 104,739 (72,348.05) |
| Sex(M) | p = 0.197 1,405.48 (1,026.48) | p = 0.266 -37.98 (47.5) | p = 0.00002 -3,925.11 (1,992.21)** | p = 0.105 -210.83 (89.16)** | p = 0.390 -80.59 (52.74) | p < 0.0001 34.92 (47.94) | p = 0.005 1.18 (0.5)** | p = 0.198 -100,925.8 (67,710.16) |
| Stress reactivity | p = 0.171 23.45 (39.5) | p = 0.424 <i>0.08 (0.05)*</i> | p = 0.049 55.27 (246.81) | p = 0.019 -0.15 (0.2) | p = 0.127 0.02 (0.08) | p = 0.467 <i>0.06 (0.03)*</i> | p = 0.018 0.01 (0.02) | p = 0.187 -138.37 (200.14) |
| Temperature (°C) | p = 0.553 -224.44 (206.13) | <i>p = 0.081</i> 0.28 (0.18) | p = 0.823 <i>246.29</i> <i>(2,181.64)</i> | p = 0.438 -3.27 (2.03) | p = 0.934 -0.91 (1.2) | <i>p = 0.061</i> 0.00 (0.21) | p = 0.523 -0.18 (0.06)*** | p = 0.516 -2,441.07 (1,730.41) |
| Log faecal CORT | p = 0.277 172.72 (71.04)** | p = 0.136 -0.21 (0.08)** | p = 0.911 -324.5 (124.32)*** | p = 0.108 -0.11 (0.32) | p = 0.447 -0.07 (0.28) | p = 0.986 -0.10 (0.07) | p = 0.003 0.11 (0.04)*** | p = 0.209 <i>-434.89</i> <i>(198.81)*</i> |
| Sex(M)*Stress reactivity | p = 0.016 -71.45 (44.09) | p = 0.014 0.1 (0.14) | p = 0.010 -94.3 (287.32) | p = 0.739 0.33 (0.23) | p = 0.792 -0.02 (-0.07) | p = 0.157 -0.1 (0.13) | p = 0.010 -0.06 (0.04) | p = 0.072 240.06 (207.75) |
| Sex(M)*Temperature (°C) | p = 0.106 279.71 (709.70) | p = 0.487 0.89 (1.13) | p = 0.743 -56.33 (2,306.48) | p = 0.148 4.94 (2.12)** | p = 0.942 1.81 (1.27) | p = 0.464 -0.86 (1.14) | p = 0.111 -0.41 (0.37) | p = 0.292 2,442 (1,637.33) |

| | | | | | | | | |
|------------------------------------------|-----------------------------|------------------|------------------------------------|---------------------|----------------------|--------------------|-----------------------|----------------------------------|
| Sex(M)*Log faecal CORT | p = 0.694 | p = 0.433 | p = 0.981 | p = 0.020 | p = 0.156 | p = 0.452 | p = 0.258 | p = 0.187 |
| | -190.42 (143.31) | 0.21 (0.27) | 527.06 (264.52)** | <i>0.98 (0.53)*</i> | 0.90 (0.45)** | 0.09 (0.30) | -0.17 (0.07)** | -252.83 (523.7) |
| | p = 0.184 | p = 0.441 | p = 0.047 | <i>p = 0.062</i> | p = 0.045 | p = 0.776 | p = 0.016 | p = 0.650 |
| Residual effect | 219173 | 1.29360 | 3288082 | 1.976 | 1.73216 | 0.479865 | 0.06666 | NA |
| Individual effect | 9560 | 0.01826 | 57787 | 0.021 | 0.03586 | 0.009911 | <0.0001 | NA |
| Observations | 4,223 | 4,223 | 4,223 | 462 | 417 | 462 | 462 | 13 |
| R ² / Adjusted R ² | | | | | | | | 0.732/0.464 |
| Akaike Inf. Crit. | 63,889,920 | 13,113.140 | 75,360.960 | 1,650.883 | 1,432.861 | 998,441 | 94,943 | |
| Note: | *p<0.1; **p<0.05; ***p<0.01 | | | | | | | |

4. Discussion

My results revealed individual differences in how stress behaviours and physiology influence song complexity (Table 15). I also found promising support for the use of non-invasive stress measurement techniques. Thermal imaging and faecal CORT both accurately reflected experimental elevation of CORT and were correlated to each other. These findings support the use of these tools in future studies and conservation programs. Individual differences in stress reactivity showed consistent patterns across tests, with more stress reactive individuals consistently showing high object neophobia and stronger responses to handling stress. Surprisingly, experimentally elevated CORT decreased activity in some measures and did not affect others. More stress reactive individuals appeared to have larger changes in behavioural response to elevated CORT levels. I found complex interactions between sex, stress reactivity and CORT treatment on measures of song complexity. Song complexity was higher in males, in stress reactive individuals, and when CORT was elevated. More stress reactive individuals also sang more overall, and CORT treatment increased all individuals' singing activity. Stress reactivity score predicted song complexity marginally better than physiological measures of CORT during baseline treatment. But when CORT was elevated, faecal CORT predicted song parameters more effectively than eye temperature and stress reactivity scores, and sex differences in song complexity were neutralised. Overall, these results illustrate that individual differences in stress reactivity have consequences for song performance and that the relationships between physiological stress, behaviour and sex are complicated (Table 15).

Table 15. Schematic summary of results. Red arrows pointing up indicates a significant positive effect was detected, dark blue arrows indicate significant negative effects. ‘♂’ indicates no effect was found. ‘♀’ and ‘♂’ indicate female and male effects. Effects were included if $p < 0.09$.

| | Eye temperature | Faecal CORT concentration | Song performance | Behavioural activity |
|--------------------------------------------|------------------------------------------------------------|-------------------------------------------------|-------------------------------------------------|--------------------------------------------------------|
| | | | Complexity* | Singing activity |
| CORT elevation | ↑ | ↑ | ↑ | ↓ Moving position and feather-ruffling |
| Sex | ⊘ | ↑♀ | ↑♂ | ⊘ |
| Stress reactivity score | ⊘ | ↑ | ↑ | ↓ † Moving position in stress reactive individuals |
| <u>Interactions</u> | | | | |
| Stress reactivity score and sex | ↑♀ Stress reactive females \geq stress reactive males | ⊘ | ↑♀ ↓♂ | ⊘ |
| CORT elevation and sex | ↑♂ CORT effect on males \geq females | ⊘ | ↑♂ CORT effect on males \geq females | ↓♂ † Moving position in stress reactive individuals |
| CORT elevation and stress reactivity score | ↓ CORT effect on stress reactive individuals | ↓ CORT effect on stress reactive individuals | ↓ CORT effect on stress reactive individuals | ⊘ |

* Song complexity overall finding was considered affected if $p < 0.1$ in more at least four parameters of song complexity

† Effect only observed when extreme values were included

4.1 Using thermal imagery to measure stress

Non-invasive measures of stress provide new avenues for research and conservation techniques but must be tested in a wide variety of situations and validated through experimental manipulation. I found that thermal imaging technology was an effective method of measuring changes in stress levels in the common myna. Individual eye temperatures showed a clear and fast response to CORT ingestion, where temperature increased by $\sim 0.64^{\circ}\text{C}$ in the first five minutes after ingestion. Eye temperatures immediately began to drop following this peak, supporting previous findings of this rapid CORT response system, where CORT is usually elevated by three minutes (Romero and Reed 2005). This fast elevation in temperature shows that CORT elevation is the mechanism behind this thermal response. These findings support the small number of previous studies that have tested this method and found eye temperature reflects current stress levels; demonstrated in blue tits (*Cyanistes caeruleus*), laying hens (*Gallus gallus domesticus*), and the little auk (*Alle alle*) (Herborn et al. 2015, Jakubas et al. 2022, Jerem et al. 2019).

Testing CORT in faecal samples also illustrates a physiological response to the elevated CORT treatment; the CORT treatment resulted in significantly increased faecal CORT concentration. Faecal samples also reflected this CORT elevation trend over an extended timeframe. Faecal samples show an individual's general stress over an extended period (a few hours), while eye temperature displays stress closer to real-time. The differences between these two non-invasive techniques demonstrate the advantages of both methods in measuring this key dynamic in the stress response system. Further, eye temperature and faecal CORT concentrations were marginally correlated in individuals, showing that these two methods reflect aspects of individual stress physiology while also providing information on how animals process CORT. Thus, they provide related and complementary data over different time spans. Alternative measurements of stress are available, that measure stress over span even longer time spans, such as feather CORT, hair CORT, tissue development, nails, claws and baleen (Gormally et al. 2020), which presents a range of methods to track stress throughout different periods of individuals lives.

The observed effects of short-term stress on eye temperature and faecal CORT simulated by CORT elevation contrast some of those observed during chronic stress in previous studies, even though, stress measurements during short-term CORT elevation mimic those during chronic stress. Unsurprisingly, measurements of faecal CORT during short-term CORT

elevation are similar to those of chronically stressed birds. Both types of stress, short-term in my study and chronic in previous, revealed higher levels of glucocorticoids than those of a comparatively non-stressed bird (Cyr and Romero 2008). Similarly, eye temperature measurements in myna showed similar increases to individuals of different species experiencing chronic stress who had elevated temperatures (Herborn et al. 2018). These similarities indicate that thermal imagery is effective for identifying long-term and short-term stress.

Females had slightly higher body temperatures overall, being 0.5°C warmer than males (in the five minutes preceding and following CORT ingestion), which is interesting given that male mynas are slightly larger (Heather 2005), and larger body sizes tend to produce more metabolic heat (Kleiber 1947). Females also had a marginally lower thermal response to CORT ingestion. I would suggest that this result is driven by females already having higher eye temperatures at baseline, which were closer to a physiological limit. In other words, females were already much warmer than males, so males increased in temperature more after CORT ingestion. It is unclear exactly why females were warmer overall than males. One explanation is that females are warmer for adaptive reasons, such as for maternal functions. Females' core body temperature in C57Bl/6 mice (*Mus musculus*) is significantly influenced by stage of the estrous cycle (Sanchez-Alavez et al. 2011), suggesting different requirements of heat throughout the reproductive cycle. In the great tit (*Parus major*) oviposition causes a distinct peak in body temperature, and metabolic heat production is directed toward the brood patch (Haftorn and Reinertsen 1982). However, my birds were singly housed and not breeding, so it is not clear if this explains the pattern I found.

Comparison with species with the same or a different mating strategy would be useful to understand whether this sex difference in temperature is related to mating system. For example, in species where males do more parental care and incubation, we might expect their thermal response to be larger than females. If this were the case, this effect may also be seasonal. Conversely, this sex difference may be driven by energy reserves or relative metabolic rates in each sex.

Females may have been more stressed than males overall, given their higher faecal CORT levels. This is discussed further in section 4.3, 'How does stress reactivity relate to physiological measurements of stress?'. Despite the sex difference at the elevated CORT treatment, eye temperature measurements due to increased stress were consistent within sexes

and within individuals. Given the substantial variation among individuals, thermal imaging technology may be most effective in estimating stress if individuals are compared to their prior temperatures. Comparing measurements within themselves will also control for variation in quality among individuals, as Jerem and colleagues (2018) found that blue tits (*Cyanistes caeruleus*) with poor health had lower eye temperatures. Using thermal imaging to measure stress is also effective outdoors (Jerem et al. 2019), making it a versatile and useful tool in the wild.

Applications for using thermal imaging

There are many opportunities for using thermal imaging to answer different questions regarding the effects of short-term stress in various species. For example, examining the stress responses of males and females with different stress reactivities to different stressors, such as taxidermy conspecifics or predators. This might show whether thermal stress response is always equivalent to individual behavioural stress responses. It would also be interesting to compare the thermal responses to stress in each sex in the breeding versus non-breeding seasons.

Thermal imaging technology can also provide a substantial advantage if used in the many different programmes monitoring bird populations in the wild. The relative success of different management intervention strategies could be assessed via stress response. For example, body temperature measures could be used to reflect whether individuals who became stressed more easily were more or less likely to breed and highlight what management changes would be necessary to improve outcomes for struggling populations. For example, if there were extreme weather conditions in a reserve, thermal imaging may be useful to identify if nesting birds were coping well or if they were becoming very stressed and may benefit from providing additional shelter temporarily.

4.2 Consistent differences in behavioural responses to stress

Neophobia and handling response provided consistent measures of stress reactivity. Each measure showed high variation among individuals, but measures were repeatable within individuals, supporting my hypothesis and previous findings in birds, including Japanese quails (*Coturnix japonica*), Adelie penguins (*Pygoscelis adeliae*), greylag geese (*Anser anser*) (Cockrem 2007, Dingemanse et al. 2010b). Additionally, neophobia and stress handling response were highly correlated, aligning with previous findings where stress

reactive zebra finches (*Taeniopygia guttata*) tend to have higher exploratory tendencies (Crino et al. 2017, Martins et al. 2007). This is interesting because exploration and neophobia are not always correlated traits, as these tendencies often depend on ecological circumstances (Mettke-Hofmann et al. 2002). Where factors such as diet often drive the degree of neophobia, as parrot species (Psittacidae) that fed predominantly on insects, rather than leaves were more neophobic (Mettke-Hofmann et al. 2002). Therefore, differences in degree of neophobia of the individual myna may also depend on individual feeding preferences, and their previous territory before they were captured. The common myna can inhabit various environments and have a wide, omnivorous diet (Global Invasive Species Database 2022), with a high tendency for exploration, and have been labelled an ‘urban exploiter’ species (Rogers et al. 2021).

Males were also slightly (although not significantly) more stress reactive overall than females, which may indicate a more general advantage for stress reactivity in males, although this may just be due to chance. Dingemanse *et al.* (2004) found the opposite dynamic acting in great tits (*Parus major*), where females were more stress reactive than males. Reactivity is also often lower in the more parental sex (Breuner et al. 2008). Common mynas are biparental and monogamous (Wilson 2013). Both sexes incubate in the myna, however, female mynas spend approximately twice as much time incubating than males, and only females develop a brood patch (Counsilman 1971). In many monogamous species, sexes often spend similar time and energy on parental care duties (Ketterson and Nolan 1994), which would fit with this pattern.

4.3 How does stress reactivity relate to physiological measurements of stress?

Sex and eye temperature

Eye temperatures were higher in more stress reactive individuals and males when CORT was elevated, but these effects also significantly interacted with other variables. Males had a larger increase in temperature after ingesting CORT, possibly because females were generally warmer than males. This temperature difference may have been because females were closer to or at the upper critical threshold of the thermoneutral zone (*sensu*, Kingma et al. 2012) while at baseline stress, and hence a larger temperature increase was observed in males when stress was elevated. This hypothesis assumes that heat produced in response to CORT elevated would not cause eye temperature to be elevated beyond the upper critical threshold (Hafez 1964). Alternatively, this sex difference may be due to males being slightly larger (as

previously discussed), producing a smaller physiological reaction to CORT ingestion; since the difference between the sexes was less pronounced during the baseline treatment.

In my study, all subjects were apparently within the thermoneutral zone, whereby the individual does not require metabolic effort to increase or decrease body temperature (Kingma et al. 2012); because no subjects were observed panting during the experiments. This would indicate that individuals were not too hot, and they would not have been too cold, as they were in a temperature-controlled room ($23 \pm 1^\circ\text{C}$).

Stress reactivity and eye temperature

When CORT was elevated, stress reactive individuals had lower eye temperatures. This observation was in contrast to what was expected, as previous studies have found strong positive correlations between CORT response and reactive behavioural tendencies, such as slower exploration, in elevated CORT great tits (Baugh et al. 2012). However, it is important to note that the great tit study examined individual ability to produce CORT in response to stressors, whereas I examined how the individuals responded to experimentally elevated CORT. Therefore, less stress reactive individuals may have been ‘less used to’ sharply increasing CORT and having a larger physiological response to CORT metabolism. This might have driven the difference between my findings and the previous ones in great tits. Otherwise, species differences driven by ecological context could have contributed to different reactive tendencies (Mettke-Hofmann et al. 2002). The hypothesis explaining this dynamic will be discussed further in the section below (4.4 How CORT affects behaviour depends on stress reactivity?).

The relationship between stress reactivity and eye temperature showed opposite relationships between the sexes, primarily during baseline CORT treatment. Stress reactive females had higher baseline eye temperatures, whereas stress reactive males had lower eye temperatures at baseline and elevated. The mechanism driving this pattern is unclear, but I speculate that being less stress reactive when male, and more reactive when female, could provide an advantage in this species. This hypothesis will be discussed further in section 4.6.1, Implications of simulated short-term stress.

Faecal CORT and eye temperature

Faecal CORT levels were also marginally higher in stress reactive individuals than less stress reactive individuals. Higher levels in baseline faecal CORT in these more stress reactive

individuals may be indicative of: naturally higher production of CORT without stimuli, individual quality (Astheimer et al. 1992, Cyr and Romero 2007), or stress caused by unidentified stimuli, such as the activity of birds in neighbouring cages (Noguera et al. 2017). However, when faecal CORT was elevated, it was very similar among individuals with different stress reactivities, indicating that the treatment brought subjects to a similar state rather than adding to their existing CORT levels.

However, females had higher faecal CORT than males at baseline and elevated CORT. This may indicate that females were more stressed than males, and therefore CORT ingestion increased by a similar margin. Females may have been experiencing more stress than males for a few reasons. Males in this species may have been more resilient to the stresses caused by captivity and perhaps were less affected by the lack of physical contact between individuals, although all mynas are highly social and usually roost colonially (Counsilman 1971). Seasonal fluctuations in CORT are natural (Rich and Romero 2001), and may have been higher in females, for example, to drive them to initiate nesting or mating rituals; Rich and Romero (2001), however, found no effects of sex on these day and seasonal CORT variations in house sparrows (*Passer domesticus*). Seasonal effects between the sexes CORT concentrations are unlikely therefore, to be driving this sex difference in faecal CORT concentration.

My results also differ from previous studies, where I found stress reactivity affected measurements of stress through eye temperature. While Baugh and others (2017b) found that baseline CORT did not differ between birds artificially selected for ‘bold’ and ‘shy’ personalities in great tits (*Parus major*), plasma CORT diverged when birds were restrained, stimulating elevation in CORT. My result may have been a novel finding in myna, or individuals may have been already experiencing some stress, e.g., from captivity or the activity of birds in neighbouring cages. Any additional stress effects may have been exacerbated in females, explaining higher faecal CORT levels, however, further research would be required to understand what is driving this trend.

4.4 How CORT affects behaviour depends on stress reactivity

Experimentally elevating CORT did not change most of the observed behaviours, other than reductions in moving position and feather-ruffling (apart from singing – see below). Behaviours observed during the manipulation of short-term CORT elevation were different to

those usually observed in birds experiencing long-term elevation of stress. Astheimer and colleagues (1992) found that white-crowned (*Zonotrichia leucophrys*) and song sparrows (*Z. melodia*) increased hopping activity and food searching behaviours when experiencing long-term stress; European starlings (*Sturnus vulgaris*) increased perch hopping in responding to a stressor in captivity (de Bruijn and Romero 2011). This perch hopping was postulated to be a displacement behaviour, similarly to beak wiping in European starlings (Bauer et al. 2011), song sparrows (*Melospiza melodia*) and American robins (*Turdus migratorius*, Evans 1984). Myna activity appeared to decrease overall for most subjects since the mean change of each behaviour type was negative. Therefore, unlike other long-term stress captivity studies, specific behaviours displayed by myna were not displaced to other behaviours by CORT treatment. Interestingly, the decrease in movements was more pronounced in males, who moved even less than females when elevated CORT.

It is currently unclear why short-term CORT elevation caused no observed change in behaviour or decreases in behaviour, except for singing. I expected the reverse because energy mobilisation is one of the primary functions of CORT. Spikes in CORT allow individuals to engage in energetically demanding behaviours such as fight or flight responses (Sapolsky et al. 2000). One possible explanation for this observed effect is that the predominant behavioural response observed was a change in singing behaviour since movement behaviours were somewhat limited in the cages. This limitation of movement may have also stimulated a different stress response, rather than fight or flight, initiating a freeze response. Although the fight, flight and freeze response is often attributed to individual differences (Roelofs 2017), context and species tendencies likely also play a large role in driving this response system. For example, if flying behaviour was always triggered when CORT was elevated, individuals would automatically respond poorly to extreme weather events rather than seeking shelter.

An alternative explanation for this behavioural freeze in response to CORT is that the perception of a stimulus initiates the behavioural stress response. For example, seeing or hearing predators, initiates the response, rather than automatically being activated by CORT and associated hormones alone. The stress response is a complex, integrated system and involves more than CORT elevation (MacDougall-Shackleton et al. 2019). The triggering of behavioural responses is linked to other neural and endocrine mechanisms to ensure an adaptive response. The activity of neighbouring birds may also influence behavioural

responses since surrounding activity can influence many behaviours, including antipredator response in brown-headed cowbirds (*Molothrus ater*, Fernández-Juricic et al. 2007).

4.5 The relationships between stress physiology, sex and singing

The total singing activity was much higher in more stress reactive individuals. This pattern was observed throughout two baseline days and during the experimental period following ingestion of both control and CORT injected mealworms. However, when CORT was elevated, all individuals' singing activity increased similarly. It is currently unclear what drives this finding, which has not been previously reported, because of so few previous studies in this area. Singing activity is a measure of quality, such as in the New Zealand tūī (*Prosthemadera novaeseelandiae*, Hill et al. 2018), and the white-crowned sparrow (*Zonotrichia leucophrys nuttalli*, Moseley et al. 2019, Robert et al. 2021); therefore, stress reactive individuals may be higher quality, since they had higher singing activity.

Therefore, higher levels of singing activity observed in stress reactive mynas may be explained by two hypotheses. Firstly, stress reactive mynas may have been 'higher quality' individuals, which is supported by comparatively higher song complexity observed in stress reactive birds (Table 15). Alternatively, stress reactive mynas were experiencing more stress at baseline, than less stress reactive individuals, which is supported by their comparatively higher faecal CORT levels. Higher overall CORT levels may have increased their song activity and complexity, as observed in the CORT manipulation experiment (Table 15).

Short-term CORT increase in singing behaviours

Again, this increased singing activity due to short-term stress contrasts with previous research that has shown that substantial life stress leads to a decrease in singing activity due to reduced energy and individual quality; seen in the European starling and the zebra finch (*Taeniopygia guttata*), among others (Buchanan et al. 2003, Casagrande et al. 2016, Spencer et al. 2003). The same contrasting result to previous studies, was identified in song complexity, as most song complexity parameters were higher in myna during elevated CORT. Previous findings on the effect of long-term stress have shown reduced features of complexity and number of song bouts in several passerine species (Buchanan et al. 2003, Gil and Gahr 2002, MacDougall-Shackleton and Spencer 2012, Spencer et al. 2003). However, these previous studies focused on the effects of chronic stress rather than short-term stress, which can cause

depleted energy reserves, suppress immune systems, and neural damage (Cyr and Romero 2007, Sapolsky et al. 2000), reducing individual quality.

In contrast, my manipulation of short-term stress displays a potentially adaptive response to a simulated stressor, driving increases in all song parameters of song complexity, except for syllable rate and diversity. Individual variation in song complexity due to stress reactivity and sex was also reduced when CORT was elevated. In other words, when individuals all received an increase in CORT, they responded with similar, highly complex songs, with similar measures of diversity, syllable switches, syllable score, bandwidth, and maximum frequency. I would speculate, therefore, that this dynamic is driven by an increase in the effort to manage a would-be ‘stressor’, such as communicating danger to conspecifics, or antagonising a subordinate individual, given that mynas are highly social and aggressive to other birds (mynas and other species, Global Invasive Species Database 2022, Wilson 2013). Female songs also increased in complexity more than males when CORT treatment was elevated. This may have been because male song was already more complex in most measures, and therefore males may be near the upper limit for complexity already. This increase in singing activity and song complexity driven by short-term stress illustrates an interesting trend that provides a novel finding in myna.

Stress profile and sex

My results showed unexpected, sex-specific effects on the relationships between CORT treatment, stress reactivity and song complexity. First, the effect of stress reactivity on song complexity parameters differed between the sexes. Males that were more stress reactive had less complex song. The negative relationship identified in males supports previous findings, where male collared flycatchers (*Ficedula albicollis*) that explored less were more active singers (Garamszegi et al. 2008); and stress reactive male song sparrows (*Melospiza melodia*) that sang less-complex songs (MacDougall-Shackleton et al. 2009, Schmidt et al. 2012). However, these patterns depend on ecological context, varying depending on life history, age, and season; for example, Grunst and Grunst (2014) found the opposite effect using the same species (song sparrows), finding stress reactive males had larger repertoires.

In contrast, females that were more stress reactive had more complex songs. Female song, in general, is very poorly understood due to decades of this behaviour being ignored by researchers (Riebel et al. 2005). Song complexity in females is even more poorly understood. And virtually nothing is known about the relationship between stress profile and female song.

This relationship between stress and song complexity has, to my knowledge, not been identified previously in females, highlighting an interesting new dynamic that contrasts with the male in the species. Therefore, this context can cause vast differences in the relative costs or benefits of being stress reactive and vary between the sexes, especially with different sex roles (Schmidt et al. 2014). I would speculate that this contrasting effect of stress personality between the sexes could be due to differing selection pressures in each sex, as observed previously (Breuner et al. 2008, Dingemanse et al. 2004). Selection pressures on physiological reactivity are often attributed to parental factors where reactivity can vary throughout the breeding season and between sex roles (Breuner et al. 2008). The birds in this study were captured before the beginning of the breeding season, although natural hormone fluctuations may have played a role in this dynamic. Male song in the tūī (*Prosthemadera novaseelandiae*) increases in complexity in the breeding season (Hill et al. 2015). These effects should be limited however, as these changes often depend on photoperiods (Rich and Romero 2001), and birds in this study were kept to a consistent 12h light cycle.

The interactions between sex and stress physiology affecting song performance further highlight how these relationships are context-dependent and vary based on species ecology and time. There is also some evidence that males and females use song in different contexts, which might drive different relationships with stress. Current literature indicates that male song is more central to mate attraction and male-male competition for mates, while female song more commonly is associated with female-female competition for resources, but not males (Riebel et al. 2005). Stress reactive females may benefit from more complex song, as it can indicate age and experience in the alpine accentor (*Prunella collaris*, Langmore et al. 1996), song sparrow (*Melospiza melodia*, Moore 2013), and superb starling (*Lamprolornis superbus*, Keen et al. 2016); and predicts breeding success in the New Zealand korimako/bellbird (*Anthornis melanura*) (Brunton et al. 2016), and in superb fairy-wrens (*Malurus cyaneus*, Cain et al. 2015). Therefore more reactive females may respond more rapidly to stimuli, for example, in polyandrous species (Riebel et al. 2019), where more complex song in females are ‘attractive’ to potential mates. But as Riebel and others (2019) emphasise, further research into the determinants and functions of female song is still imperative to understanding how context, such as environment types, seasonal effects, condition dependence and mating strategies, affect this ecologically significant behaviour, that is still relatively unexplored.

Despite these contrasting effects of stress reactivity on song complexity between the sexes, stress reactive individuals had more complex songs. This was observed for most song parameters, except for syllable diversity, which was lower in stress reactive individuals; and syllable rate, which showed no effects. This pattern indicates that the influence of stress reactivity on song complexity was primarily driven by females and weaker in males. Therefore, stress reactivity may not be detrimental for song performance in males but likely derives benefits in females, as discussed. Future research into the exact function of female song in this species in the wild may highlight what drives this trend in females that are not observed in males.

The effect of sex on song

Male songs were more complex than female song, in most measures. This observation may be driven by a variety of circumstances acting on myna. For example, song divergence between the sexes has mostly been assumed sexual selection on males (Riebel et al. 2005). But sexual song divergence could also be derived from selective advantages of certain song types between the sexes. For example, selection for contrasting functions of the songs between the sexes (Riebel et al. 2019).

Further, spectral flux showed the most pronounced sexual dimorphism; the maximum spectral flux in females is much lower than the maximum flux in males, unlike other song parameters. One potential explanation of this is a physiological limit to fast changes in frequency caused by physical differences such as in the syrinx or the brain's higher vocal centre (HVC). The size of the HVC is a strong indicator of vocal complexity (MacDougall-Shackleton and Ball 1999). Although male and female starlings (*Sturnus vulgaris*), mynas' close relatives, have similar HVC sizes, both sexes also have complex songs (Ball 2016). However, red-backed fairy-wrens (*Malurus melanocephalus*) have clear differences in brain anatomy between the sexes, but have equal singing activity and subtly different but equally complex song between the sexes (Schwabl et al. 2015). Female and male song divergence can also be driven by a decoupling of learning strategies, where females often show more variation in song structure than males (Mennill et al. 2006). In myna, these sex differences were only moderately diverged, and substantial overlap existed in song complexity. Potential explanations of these patterns would require further investigation into selection pressures and fitness outcomes that drive these differences.

Male and female songs did not differ in syllable diversity, even though syllable diversity was affected by individual stress reactivity. Therefore, males may sing the same syllables as females, but sing them ‘better’. For example, in the ‘sweep’ syllables, males may sweep up or down in frequency faster than females are physiologically capable of.

General song observations

Relationships for song syllables bandwidth were opposite to those of most other song parameters, like syllable score, syllable switches and maximum frequency (Fig. 17).

Bandwidth likely showed opposing trends because more complex syllable types scored higher and had higher spectral flux, such as ‘wave’ which covered a smaller frequency range than most other syllable types. However, simpler syllables like ‘buzz’ had more ‘noise’ and covered a larger bandwidth and do not follow a clear pattern. High bandwidth can also occur with the loss of stereotypy in syllables, meaning they are ‘messier’ than others. Therefore, a lower bandwidth may describe mynas’ more complex song type. Syllable rate was not affected by any metric and may not be an important feature of song complexity in this species; Goller (2022) proposed that individual singers (within or between species) specialise in singing certain features of song, rather than being outstanding in every measure of song complexity. For example, in the rufous-and-white wren (*Thryothorus rufalbus*), male bachelors sing more songs with a higher syllable diversity, but performed a smaller repertoire than paired males, suggesting a change in singing strategy (Hennin et al. 2009). Thus, mynas may display quality by including song features with fast frequency sweeps and complex syllables rather than singing particularly fast.

4.5.1 Which measure of stress best explains song complexity

The physiological measures that best-explained song complexity depended on whether birds were in baseline versus elevated CORT conditions. In baseline conditions, an individual’s stress reactivity (a composite measure of how they responded to novel objects and handling) was the best predictor of variation in song complexity. Individual reactivity was related to three different song parameters and was at least marginally important for six measures. Eye temperature explained three measures the best, one being marginal. Faecal CORT was the least effective measure at predicting song complexity, only explaining one parameter the best and marginally explaining two. In contrast, when stress was elevated, faecal CORT explained song complexity most effectively, explaining five parameters the best and seven parameters

at least marginally. Temperature explained two parameters the best, stress reactivity explained one measure the best, and three at least marginally.

Individual song complexity appears to be well explained by eye temperature measurements and faecal CORT. Due to these similarities observed and how well faecal CORT explained measures of song complexity when CORT was elevated, I would suggest that eye temperature predicted song complexity less than faecal CORT. Eye temperature and overall song complexity had opposing trends in each sex, despite other patterns being similarly reflected in the two metrics. This exhibits how useful these non-invasive technologies are, as the appropriate method can be chosen to best complement the research question.

As with most of my findings, measures of song complexity had sex-specific relationships. When stress was at baseline, sex significantly affected six of the eight song complexity parameters, while it only affected three at elevated. Thus, the sexes show differing relationships between complexity measures and reactivity during baseline conditions, but the sexes were more similar when CORT was elevated.

Both non-invasive methods predicted parameters of song complexity to some degree, but faecal CORT was the best predictor at elevated CORT. Overall, stress reactivity is an important element influencing song performance, especially regarding total singing activity. The way stress reactivity influences singing activity may be indicating that individuals are experiencing agitation rather than strongly reflecting individual quality, since stress reactivity did not overwhelmingly predict song complexity. However, these trends are not evident when stress was elevated; as song quality was relatively uniform among individuals at elevated CORT.

4.6 Synthesis

4.6.1 Implications of simulated short-term stress

The effect of CORT on singing

My findings supported the hypothesis that singing activity would increase, but in contrast to my hypothesis, CORT treatment also increased song complexity. This may indicate that the activation of energy reserves in responding to CORT (Romero and Beattie 2021) provides individuals more energy to put more effort into their song, thereby increasing its quality from baseline. Another possible explanation is individuals still have plentiful energy reserves and experience an increased demand for complex song as presumably, a ‘better’ song is required

when individuals are responding to a stressor. An example would be responding to challenges from conspecifics in the ‘challenge hypothesis’. Most research on the hormonal response to intruders has focused on an increase in testosterone in the ‘challenge hypothesis’ (Goymann et al. 2007, Wingfield et al. 1990), where males with increased testosterone fair better in mate rivalry challenges (Wingfield et al. 1990). Although testosterone is important to male songs, this effect is not observed in females (Ball 2016). However, this short-term elevation in CORT used here may mimic a hormonal response to an intruder in the territory when it would be advantageous to increase song complexity. CORT increases much more quickly than testosterone, and it could outline an important mechanism for such short-term challenges.

An increase in song quality due to short-term stress would be a beneficial adaptation since song complexity is an honest signal of quality (Gil and Gahr 2002). Therefore, increased song quality provides stronger effects, such as conveying fitness, deterring territory intruders, or displaying higher quality to mate challengers in either sex (Cain et al. 2015, Kipper et al. 2006, Riebel et al. 2019). But excessive stress activation and decreasing fitness would decrease song quality over time. Hence, there is evidence that long-term stress activations decrease song quality as previously discussed (Buchanan et al. 2003, Casagrande et al. 2016, Spencer et al. 2003).

It would be interesting to further investigate the factors that drive this increased singing activity and song complexity in response to CORT elevation. For example, if this experiment was repeated in different species, such as a closed-ended learner. Would the same plasticity in song complexity and singing activity be observed in response to elevated CORT? This may indicate whether increased song complexity is driven by increased ‘effort’ or perhaps a neural mechanism otherwise if the same response was observed, increased song quality may be an innate response to elevated CORT. Therefore, a universal adaptive response to an activated hypothalamus-pituitary-axis (HPA) may be evident across all songbirds. Otherwise, this increase in song quality may be a derived response, potentially found in species that rely more heavily on complex song, for survival, communication, defence and other functions. Therefore, further research into song changes in response to stress in different species and the wild may provide a further understanding of this response.

Physiological response to CORT elevation

The physiological changes caused by a simulated short-term stressor are highlighted in this

study. Eye temperature rapidly increased and was regulated within ten minutes. However, faecal CORT patterns showed this change in ‘stress status’ over one to two hours. This rapid temperature change may be due to fast-acting thermoregulation, explained by the ‘thermoprotective hypothesis’. This hypothesis predicts that the function of stress-induced increases in body temperature is to aid thermoregulation (Robertson et al. 2020, 2021). This hypothesis would explain the comparatively extended effects of CORT ingestion in faecal CORT and song parameters. These lasting effects in song complexity may indicate that ingestion of CORT had lasting effects, potentially activating a response to a would-be stressor, even if the initial rise in CORT was regulated already. Having a lasting response to any rise in CORT is likely to be an adaptive response, as it would allow individuals to produce higher quality responses (such as a high-quality song) without continual production of CORT. For example, in a mate rivalry contest, an initial rise in CORT would stimulate a lasting increase in song quality, providing an advantage in gaining mates. This allows an adaptive advantage in moderately stressful situations without potentially becoming chronically stressed every time a conspecific is encountered.

The behavioural response to short-term stress contrasted with long-term stress in previous studies. Although this difference in response is likely to be adaptive, a behavioural response should be appropriate to the stressor, not a universal stress response. However, freezing in response to elevated CORT may be an adaptive response as well, although freezing behaviour is not a typical behavioural response in the myna (Counsilman 1971). Factors such as prior experiences and individual stress reactivity may determine the intensity of responses when in context. However, understanding this behavioural stress response further requires testing for behavioural responses to stressful stimuli in context to identify trends in certain responses that arise to different stressors.

Non-invasive measurement

Differences between faecal CORT and eye temperature further highlight how these two non-invasive methods of measuring stress can be chosen to best suit the situation. For example, eye temperature is useful and effective for rapid responses to stress, such as in hand-rearing or during stressful interventions like translocations. Herborn (2018) and colleagues indicate that thermal imaging reflects longer-term CORT elevation in laying hens (*Gallus gallus domesticus*). While, faecal CORT may be useful for longer-term studies, looking at a population or individual stress throughout or between seasons. This population monitoring method is more effective than plasma samples as it reflects consistent increases in

glucocorticoids in response to anthropogenic disturbances, unlike plasma (Dantzer et al. 2014). It is important to note that eye temperature measurements vary among individuals. Especially since individual energy reserves can be reflected with thermal cameras (Jerem et al. 2018), this technology appears most accurate when individuals are compared to themselves.

Although identifying chronic stress in populations being monitored may present more of a barrier when relying on any measure of CORT, chronic stress often presents differently in baseline CORT levels, sometimes being higher, lower or having no effect (Dickens and Romero 2013). Monitoring individual weight may be a more effective method for specifically identifying signs of chronic stress (Dickens et al. 2009, Dickens and Romero 2013). Faecal CORT may also be a more useful measure for future studies investigating stress and song complexity since this was the most useful parameter when CORT was elevated. Further studies into using eye temperature and faecal CORT together in different contexts and species is essential to compare these methods. This would help build a fuller understanding of the applications that thermal imaging can have; this rapid method may be a better way of measuring a population's response to disturbances such as anthropogenic or predatory difficulties. This new method still has many unanswered questions but appears to be a promising new technology for conservation practices, as discussed in section 4.1, 'Using thermal imagery to measure stress'.

Limitations

Given this study was conducted in captivity, most variables affecting results were controlled for, but some limitations could not be avoided. It cannot be ruled out that birds were experiencing extended periods of stress prior to experimentation, living in captivity and having researchers nearby each day. Stress reactive individuals had higher faecal CORT concentrations, and stress-induced by captivity may have contributed to this. The birds were monitored closely for signs of chronic stress, such as decreased body weight, over preening or other (Blas 2015, Dickens and Romero 2013). Further, no previous studies have investigated differential individual stress caused by captivity, linked with stress reactivity. However, the benefits of conducting this study in captivity outweigh the limitations, as temperature, sound, and other potential stressors and stimuli could all be controlled.

Regarding the thermal experiment, any heat produced due to metabolic rise during digestion of the mealworm was not controlled for. Although the specific dynamic action (SDA), or heat

increment of feeding duration and response, is most strongly influenced by the type, composition, temperature, and size (relative to the body) of the meal ingested (Secor 2009). The single mealworms fed during this experiment had no exoskeleton, were frozen, and were a maximum of 0.0009% of the myna body volume, providing around 1.38kJ of energy. Therefore, this meal, relative to body size and energy provided, was likely negligible. Although best practice would have been to control for this and repeat the test with the mealworm only, I was unable to due to logistical constraints.

4.6.2 Implications of stress reactivity

The effects of short-term stress differed among individuals with different stress reactivities. I identified effects of stress reactivity with individual eye temperature, singing behaviour and complexity, but no obvious effects on other behaviours. These similarities outline that eye temperature appears to reflect individual song complexity well for many chosen song parameters. Individual ‘quality’ differences may also drive these similarities. As song quality and activity parameters positively correlate with individual fitness or health (Cain and Ketterson 2012, Gil and Gahr 2002). But the same is true for measures of eye temperature, as birds with lower body condition were found to have lower eye temperature in wild blue tits (*Cyanistes caeruleus*) (Jerem et al. 2018). Therefore, individuals measuring higher may also have more energy reserves or be healthier individuals. Individuals with higher baseline temperatures, being more stress reactive females and less stress reactive males, may be healthier individuals. This may indicate an advantage for different reactivities in each sex, as has been previously observed (Breuner et al. 2008, Dingemanse et al. 2004). Stress reactivity can also vary between and throughout the seasons, in response to health conditions, life, and day (Breuner et al. 2008).

The relative benefits derived from being more or less stress reactive are highly dependent on each species or population (Taborsky et al. 2021). Factors such as location, including latitude, can influence how beneficial being stress reactive is (Breuner et al. 2008). Future research should consider different factors influencing the species ecology, such as weather conditions and species mating style, as these are likely to influence the relative benefits of being stress reactive. For example, in the common myna, if and how individuals benefit from being more or less stress reactive is unclear without further research. Identifying direct fitness benefits in each sex, in context, is required to unravel the drivers behind these specific patterns.

However, predictability of environment is thought to play a substantial role in shaping the evolution of the stress response. In contrast, Taborsky and others (2021) summarise several hypotheses, including one postulating that CORT reactivity creates a trade-off, aiding survival and impairing reproduction. These relationships between stress reactivity and adaptation can also differ between the sexes, as seen in my results on song complexity (Table 15). Male and female great tits (*Parus major*) contrast in which strategy is more beneficial, depending on resource availability (Dingemanse et al. 2004). Therefore, plasticity in an individual's behavioural stress response can provide an enhanced ability to adapt to different ecological circumstances (Bonier and Martin 2016, Montiglio et al. 2018). Cockrem (2013) proposed that individuals who were more 'shy' and more stress reactive are likely to have better chances of survival in climate change; being less likely to take dangerous risks when environments significantly change. However, Bonier and Martin (2016) suggest that plasticity in stress response is advantageous and allows continued adaptation throughout changing environments. Having rapid and effective responses to significant environmental perturbations will greatly advantage the chances of survival as climate change begins to disrupt otherwise stable ecosystems (Wingfield 2013). Research into different species' ability to have this flexibility in response system is imperative to understand which species are the most at risk under these changes.

Limitations

Some interactions that may affect the measurement of stress or behaviour could also have interfered with these measurements. Stress from birds in neighbouring cages could influence an individual's physiological and behavioural stress, as this influences individual's stress in the yellow-legged gull (*Larus michahellis*) (Noguera et al. 2017). Further, behaviours or vocalisations that may communicate stress could have influenced individuals to respond in similar ways. These influences could not be avoided since the common myna is a social species and needed to be co-housed with other individuals for their well-being.

4.6.3 Implications of sex differences

This study highlighted many differences in the relationships between stress and song performance. Male song was more complex at baseline, but female song was equally complex when elevated, and singing activity was equal between the sexes. Therefore, song is an important behaviour for both sexes but perhaps serves different functions due to the divergence in complexity observed at baseline CORT. Indicating that whatever the function

of female song, a highly complex song is still achievable and potentially advantageous in this species. Differences in song complexity between the sexes have many possible explanations. For example, if females had had lower maximum spectral flux because of physical differences in their syrinx, as previously suggested, this may be explained by a few theories. Physical differences could be due to an ancestral state of male and female song, both having similar spectral flux that is now diverging; it is unlikely to be converging since female song is the ancestral state (Odom et al. 2014). Otherwise, a benefit may be currently derived from males having faster frequency sweeps and more complex, driven by a stronger selection in males than females.

Differences in birdsong's acoustic structure between the sexes are often driven by differences in the function of each sexes song (Sierro et al. 2021). Songs used only for communication, compared to songs that display quality, like in mate attraction and female-female competition, tend to be less elaborate (Hall et al. 2015). Therefore, I would predict that female song in this species does not function only in communication, but the complexity indicates a function in displaying quality. Female song in this species is also likely to be a multipurpose trait, as is usually the case for male song, such as in superb fairy-wrens (*Malurus cyaneus*), functioning strongly in female-female competition (Cain and Langmore 2015). However, female song has many documented functions, including male-female communication, female-female communication, territory defence, competition for resources, mate attraction and mate guarding in monogamous species (Riebel et al. 2019).

I would predict that at least one function of female song in common mynas would be in territory defence, or mate defence. Female starlings (*Sturnus vulgaris*, close relatives of myna) produce more songs when using nest boxes but sing less as the breeding season progresses and sing less around males (Pinxten et al. 2007). If female myna song is similar to starlings, this would indicate some territory defence. Furthermore, common mynas are highly monogamous (Wilson 2013) and are unlikely to have strong male sexual selection and divergence in song elaboration since females are capable of a similarly complex song (Odom et al. 2021). Despite this strong monogamy, mynas produce the most song during the breeding season; functioning in territory defence, pair formation, and parents often produce loud trills when approaching the nest (Craig and Feare 2010). Although it is unclear how much each sex produces songs in these contexts. But to identify the various functions of female song in this species, specific research in the wild is required, testing for fitness benefits derived by each sex by performing better quality songs in different contexts.

Future research

These divergences between the sexes when stress is at baseline raise interesting questions about what differences are driving them. Further investigation into drivers of female song in this species may highlight more trends indicating its function. Future research may also tie in how vocal mimicry functions in this species and how much each sex displays. The function of avian vocal mimicry is highly contested, proposing evolutionary convergence (Kelley et al. 2008) or deception (Wickler 2013), but also often filling multiple functions like other vocalisations (Dalziel et al. 2015). Therefore, studies that can identify how much each sex displays it may highlight its functions and perhaps shed more light on this issue for other species showing vocal mimicry. The common myna has proven to be an interesting model species, showing complex vocalisations in both sexes. Common mynas are also very successful invader species (Magory Cohen et al. 2019) and are very aggressive and dominant where they are found (Counsilman 1971). So future studies may highlight whether their vocalisations contribute to their success and may enlighten what makes invasive species so successful. Many countries are currently struggling to manage or eradicate populations of the common myna now (Global Invasive Species Database 2022). Therefore, these overall trends observed driving differences in vocalisation behaviours have implications for successfully managing this species as a pest.

4.7 Conclusions / Future directions

This study highlights the complex interactions in the stress response system and how it affects vocalisations and behaviour, driven by stress reactivities and sex. I first identified that simulated short-term stress strongly impacts song complexity in the common myna, which has not been identified previously. Simulated short-term stress increased singing activity and most parameters of song complexity. These effects contrasted previous findings of long-term stress and highlighted what are likely adaptive functions of the stress response in all individuals; individuals may benefit from performing better songs when in stressful situations such as deterring territory intruders. Behaviours expressed during stress are likely driven by context, choosing an appropriate response to a stimulus rather than CORT alone. Further research in the wild should investigate direct or indirect fitness benefits gained from increasing song complexity in response to stress.

The effects of simulated short-term stress differed among individuals with different stress reactivities. Singing activity and song complexity were higher in individuals who were more

stress reactive, and this effect was stronger in females than males. Stress reactivity was found to play an important role in many of the observed dynamics. The patterns observed in this study have real implications for individual fitness. Individual personalities and reactivities of birds affect the way they respond to stressors in the wild and could affect their ability to attract mates or defend territories. Research has shown that increased stress in native bird populations significantly impacts conservation efforts (Harding et al. 2019). Therefore, understanding how individuals respond differently to stress is essential to understanding how best to protect threatened species and should be considered in future studies, especially regarding vocalisations.

This study highlights interesting sex-specific trends in song performance; females' song complexity increased more than males after CORT elevation, and females drove the positive effect of stress reactivity on song complexity. Females may benefit more from having higher plasticity in song performance, while males may require more consistent song complexity.

Further research into sex-specific selection on song complexity and plasticity would help understand this trend further, especially seeing if there are direct fitness benefits between the sexes in responding to stressful events. Effects of sex-specific selection on song may also vary in different species, for example, with different mating strategies or in different environmental stabilities; researching species in different contexts may, therefore, highlight how different circumstances drive a sex-specific selection of stress on song.

Further research into the functions of female song, in this species and others would provide valuable insights into this important phenomenon that has been overlooked. These studies would need to test for real fitness benefits derived from having complex song displayed in different contexts. Future studies may investigate sex differences in vocal mimicry in the myna and what drives mynas' global success.

Thermal imaging has proven to be a useful non-invasive method of measuring stress and can be used with faecal CORT to identify stress dynamics. Thermal cameras appear to be most effective when compared within individuals; however, individual quality, sex, and personality contribute to differences in temperature readings. Other than contrasting effects observed between the sexes, stress reactivity and CORT affected eye temperature and vocal complexity in similar ways. However, when CORT was elevated, faecal CORT predicts vocal complexity the best.

Understanding how stress personality plays a role in the common myna is an important outcome, as this species is also a significant pest undergoing management in many countries (Global Invasive Species Database 2022, Magory Cohen et al. 2019, Parkes and Avarua 2006, Tindall et al. 2007). Although understanding the ecology of a species is undoubtedly vital in successful species management programmes, the actions of individuals that behave very differently from the average are the most important and challenging to identify and manage (Garvey et al. 2020). For example, a few individuals in this study had incredibly low singing activity compared to others. Therefore, if common myna eradication programmes relied heavily on vocalisations to locate individuals, these individuals would very likely be missed. Thus, it is important to incorporate individual differences in future studies, as it plays large roles in behaviours like vocalisations and highlights important considerations to make in practices such as pest species management.

Understanding these patterns shown is likely to become more critical in the context of Aotearoa, New Zealand as the common myna has previously been found to produce strong competition with native cavity-nesting species in Australia, with the crimson rosella (*Platycercus elegans*) and the eastern rosella (*Platycercus eximius*) (Grarock et al. 2013). The endemic cavity-nesting bird species in New Zealand already experience significant threats due to competition and predation (Parlato et al. 2015, Rhodes et al. 2009). Therefore, knowing the behaviours of individuals that show more extreme tendencies may be beneficial for management programmes in New Zealand if the common myna becomes more of a threat to native avifauna.

Therefore, understanding how individual reactivities affect how resilient individuals are to perturbations is essential. Factors like anthropogenic disturbances have significantly affected a range of species, impacting their ability to survive (Kunc and Schmidt 2019). For example, even just anthropogenic noise can drastically alter birdsong, and changes that may be necessary for survival, are not achievable in all species (Slabbekoorn and Ripmeester 2008). Further research into individual adaptability is essential, especially because significant changes and disturbances to major habitats will only increase with climate change, and understanding the suitability of different stress personalities can only help manage this pressing issue (Wingfield 2013). Consequently, understanding these effects, particularly differences between the sexes, can allow management to prioritise resources to support differentially reactive individuals, ensuring the long-term effects of stressors do not have lasting, devastating effects on populations.

5. Supplementary

Supplementary Table 1. Summary of a linear mixed model (LMM) results showing the time taken to complete neophobia tests relationship handling response score. The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold.

| Time taken in neophobia tests | | | | |
|------------------------------------------------------|------------------|------------------|------------------|--------------|
| <i>Predictors</i> | <i>Estimates</i> | <i>CI</i> | <i>Statistic</i> | <i>p</i> |
| (Intercept) | -186.00 | -541.51 – 169.51 | -1.03 | 0.305 |
| Rating | 137.78 | 27.04 – 248.51 | 2.44 | 0.015 |
| Random Effects | | | | |
| Residual effect | 16368.50 | | | |
| Individual effect | 64982.71 | | | |
| N _{Subject} | 13 | | | |
| Observations | 26 | | | |
| Marginal R ² / Conditional R ² | 0.300 / 0.859 | | | |
| AIC | 340.163 | | | |

Supplementary Table 2. Summary of variance attributed to each variable included in the principal component analysis of stress reactivity.

| <i>Variable</i> | <i>Variable loadings</i> | |
|------------------------|--------------------------|----------------|
| | <i>PC1 (%)</i> | <i>PC2 (%)</i> |
| Stress rating 1 | 30.37 | 14.28 |
| Stress rating 2 | 32.31 | 10.73 |
| Time taken in N1 | 28.08 | 7.46 |
| Time taken in N2 | 9.24 | 67.54 |
| Variance explained (%) | 59.9 | 25.5 |
| Eigenvalues | 2.4 | 1.01 |

Supplementary Table 3. Summary of linear mixed models (LMM) predicting how parameters of song complexity are affected by interactions of stress reactivity, CORT treatment (baseline: where CORT was not altered and elevated: after ingestion of mealworm injected with 20ug of CORT), and sex(m/f). Estimate, standard error and significance are presented for each fixed effect. The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold, and $P < 0.1$ are indicated in italics.

| | <i>Dependent variable:</i> | | | |
|----------------------------------------------|----------------------------|-------------------------|-------------------------|--------------------------|
| | syllable rate | syllable diversity | syllable switches | syllable score |
| Intercept | 3.668 (0.317)*** | 0.584 (0.061)*** | 1.077 (0.321)*** | 1.736 (0.160)*** |
| Stress reactivity | p < 0.0001 | p < 0.0001 | p = 0.001 | p < 0.0001 |
| | 0.036 (0.186) | -0.089 (0.036)** | 0.460 (0.188)** | 0.197 (0.092)** |
| Treatment(Elevated) | p = 0.848 | p = 0.013 | p = 0.015 | p = 0.032 |
| | 0.178 (0.321) | -0.043 (0.060) | 0.805 (0.317)** | 0.490 (0.165)*** |
| Sex(M) | p = 0.580 | p = 0.474 | p = 0.012 | p = 0.003 |
| | 0.106 (0.355) | -0.052 (0.069) | <i>0.711 (0.363)*</i> | 0.546 (0.178)*** |
| Stress reactivity*Treatment(Elevated) | p = 0.767 | p = 0.453 | <i>p = 0.051</i> | p = 0.003 |
| | 0.054 (0.182) | 0.085 (0.034)** | <i>-0.340 (0.179)*</i> | -0.119 (0.093) |
| Stress reactivity*Sex(M) | p = 0.767 | p = 0.012 | <i>p = 0.058</i> | p = 0.202 |
| | -0.186 (0.219) | 0.055 (0.043) | -0.524 (0.223)** | -0.281 (0.108)*** |
| Treatment(Elevated)*Sex(M) | p = 0.397 | p = 0.194 | p = 0.019 | p = 0.010 |
| | 0.232 (0.354) | -0.006 (0.066) | <i>-0.589 (0.351)*</i> | -0.367 (0.182)** |
| Stress reactivity*Treatment(Elevated)*Sex(M) | p = 0.513 | p = 0.933 | <i>p = 0.094</i> | p = 0.045 |
| | -0.002 (0.215) | -0.028 (0.040) | 0.328 (0.212) | 0.228 (0.110)** |
| | p = 0.992 | p = 0.481 | p = 0.122 | p = 0.039 |
| Residual effect | 1.618 | 0.066 | 1.853 | 0.504 |
| Individual effect | 0.062 | 0.003 | 0.084 | 0.012 |
| Observations | 681 | 744 | 744 | 744 |
| Akaike Inf. Crit. | 2,302.124 | 158.841 | 2,613.429 | 1,651.648 |
| <i>Note:</i> | | | | |
| *p<0.1; **p<0.05; ***p<0.01 | | | | |

Supplementary Table 4. Summary of linear mixed models (LMM) predicting how parameters of song complexity are affected by interactions of stress reactivity, CORT treatment (baseline: where CORT was not altered and elevated: after ingestion of mealworm injected with 20ug of CORT.), and sex(m/f). Estimate, standard error and significance are presented for each fixed effect. The variance attributed to the random effects of Subject ID and the residual are presented for the model. Significant parameter estimates, where $P < 0.05$, are highlighted in bold, and $P < 0.1$ are indicated in italics.

| | <i>Dependent variable:</i> | | |
|----------------------------------------------|------------------------------|-------------------------|-------------------------------|
| | Bandwidth (Hz) | Spectral flux | Maximum frequency (Hz) |
| Intercept | 3,540.538 (86.036)*** | 0.403 (0.166)** | 989.719 (275.659)*** |
| Stress reactivity | p < 0.0001 | p = 0.016 | p = 0.0004 |
| | -97.098 (54.133)* | 0.055 (0.102) | 426.675 (165.263)*** |
| Treatment(Elevated) | p = 0.073 | p = 0.589 | p = 0.010 |
| | -322.447 (63.489)*** | 0.450 (0.140)*** | 1,129.689 (246.027)*** |
| Sex(M) | p < 0.0001 | p = 0.002 | p = 0.00001 |
| | -303.062 (102.746)*** | 0.432 (0.191)** | 1,125.511 (308.763)*** |
| Stress reactivity*Treatment(Elevated) | p = 0.004 | p = 0.024 | p = 0.0003 |
| | 110.270 (35.001)*** | 0.027 (0.077) | -301.162 (135.497)** |
| Stress reactivity*Sex(M) | p = 0.002 | p = 0.731 | p = 0.027 |
| | 176.328 (64.957)*** | -0.153 (0.118) | -583.243 (189.486)*** |
| Treatment(Elevated)*Sex(M) | p = 0.007 | p = 0.198 | p = 0.003 |
| | 265.553 (66.212)*** | <i>-0.248 (0.146)*</i> | -931.579 (256.586)*** |
| Stress reactivity*Treatment(Elevated)*Sex(M) | p = 0.0001 | p = 0.090 | p = 0.0003 |
| | -190.020 (38.146)*** | 0.065 (0.084) | 381.167 (147.725)*** |
| | p < 0.0001 | p = 0.441 | p = 0.010 |
| Residual effect | 219719 | 1.07830 | 3316253 |
| Individual effect | 18344 | 0.04657 | 97510 |
| Observations | 6,863 | 6,863 | 6,863 |
| Akaike Inf. Crit. | 103,875.900 | 20,070.070 | 122,473.700 |
| Note: | *p<0.1; **p<0.05; ***p<0.01 | | |

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