Restraint increases afebrile body temperature but attenuates fever in Pekin ducks (*Anas platyrhynchos*).

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Abstract

In mammals, procedures such as handling, restraint, or exposure to open spaces induces an increase in body temperature ($T_b$). The increase in temperature shares some characteristics with pyrogen induced fever, and so is often called “stress fever”. Birds also respond to acute handling with a stress fever, which may confound thermoregulatory studies that involve animal restraint. We have measured the $T_b$ responses of Pekin ducks on days when they were restrained and compared them to days when the birds remained unrestrained. Restraint induced a 0.5°C increase in $T_b$ that was sustained for the entire 8 hours of restraint. To determine if the restraint-induced increase in $T_b$ is mediated by prostaglandins (PGs) we compared the $T_b$ responses during restraint after intraperitoneal injection with saline to the responses during restraint after injection with diclofenac sodium (15 mg/kg). There was no difference in response, suggesting that restraint affects $T_b$ by a prostaglandin independent mechanism. We also compared the $T_b$ response to intramuscular injection of lipopolysaccharide (LPS, 100 µg/kg), a bacterial pyrogen, when the ducks were restrained or unrestrained. Despite $T_b$ being higher at the time of LPS injection when the ducks were restrained, the maximum temperature reached after LPS injection was higher, and the period that $T_b$ remained elevated was longer, when the ducks were unrestrained. We conclude that restraint should be considered as a potential confounder in thermoregulatory studies in birds and presumably other species too.
Introduction

A comprehensive understanding of the phylogeny of the febrile response requires a detailed knowledge about fever in species other than mammals and in particular the other group of homeotherms, birds. There have been several studies in a variety of avian species characterizing the febrile response to pyrogens (9,17,20,21) and evidence indicates that mammalian and avian fever share significant functional similarities with respect to mediation and modulation (7,9,12,14,21,25). However, our understanding about other aspects of the physiology of fever in birds remains extremely limited in comparison to mammals.

In mammals, psychological stress results in an elevation of body temperature ($T_b$), so that procedures such as handling (3,4,31), restraint (24,30), and exposure to open spaces (14,25) or novel environments (17) induce “stress hyperthermia”. There is some evidence that this stress-induced rise in $T_b$ is in fact a fever, since inhibition of some of the known mediators of fever, such as prostaglandins (PGs) (28) or nitric oxide (NO) (29), inhibit the increase in $T_b$. A psychological influence on $T_b$ also seems to be present in birds, since $T_b$ increases in a variety of avian species in response to handling (6,12,17). In chickens, the handling-induced elevation of $T_b$ is inhibited by salicylate (6), a known inhibitor of fever, suggesting that avian stress hyperthermia also exhibits some of the characteristics of fever. But our understanding of the mechanism(s) underlying the $T_b$ response to psychological stress in birds, or the effect of stress on the fever response to pyrogen, is far from clear. In particular clarity is needed about how physical restraint influences
thermal biology. Physical restraint is used in many mechanistic studies, for example, when regular blood sampling is required to measure the endocrine mediators of fever (10,11,17). If restraint induces a psychological stress resulting in elevated $T_b$ or fever, then results will be confounded by that response. Indeed we are unaware of studies, even in mammals, that have examined whether superimposing a psychological stress on a fever alters the thermal response to the pyrogen. Therefore the present study was designed with two objectives: i) to determine if sustained physical restraint has a more than transient effect upon normal $T_b$ and, if so, whether this restraint-induced effect is affected by the inhibition of PGs, known mediators of avian fever (12) and ii) to determine whether stress-induced changes in $T_b$ alter the time course and/or magnitude of a superimposed febrile response induced by lipopolysaccharide (LPS).

To accomplish these objectives we carried out two series of experiments in adult Pekin ducks, an avian species that has been used frequently in thermoregulatory studies (10,11,12,17) and for which an extensive physiological data base exists. In the first series we compared the $T_b$ profiles of ducks when they were unrestrained to when they were held in restraints for eight hours, a time course that covers the normal avian fever response to LPS. We also determined the effect of diclofenac sodium, a known inhibitor of PG formation and the febrile response in Pekin ducks (12), on the $T_b$ profiles of the ducks when they were restrained. In the second series we compared the characteristics of the febrile response to lipopolysaccharide (LPS), an
established pyrogen in Pekin ducks (10,11,12,17), in restrained and unrestrained animals.

Methods

Animals

Ten (4 male, 6 female) adult Pekin ducks (Anas platyrhynchos) within a weight range of 2.4 – 3.2 kg, housed in flocks at a room temperature of 22°C and with a natural day-night cycle (sunrise (median) 05:13 local time; sunset (median) 18:32 local time; day length (median) 13.3 ), were used. They were fed dry chicken food enriched with minerals and vitamins, and drank tap water ad libitum. The study was approved by the Animal Ethics Committee of the University of the Witwatersrand (clearance number 2006/42/04).

Body temperature measurement

Core temperature of the ducks was recorded at 10-min intervals using wax-coated miniature temperature data loggers (iButton, Maxim Integrated Products, Sunnyvale, CA) implanted into the abdominal cavity. Data logger implantation was done under 2% isoflurane (Isofor, Safe Line Pharmaceuticals, Johannesburg, RSA) anaesthesia at least 10 days before experimentation. Before implantation, data loggers were calibrated over a range of temperatures (34 to 44°C at 2°C intervals) against a certified precision thermometer (Quat 100, Heraeus, Hanau, Germany) such that abdominal temperature was measured to an accuracy of 0.1°C.
Pyrogens and drugs

A stock solution of LPS (Salmonella typhosa, Sigma, St Louis, MO) was prepared in sterile saline, such that appropriate doses could be administered in an injected volume of 1 ml/kg. The cyclooxygenase inhibitor diclofenac was obtained as a commercial preparation in sterile ampoules containing 75 mg diclofenac sodium in 3 ml sterile saline with 2% (v/v) ethanol as solvent (Merck, Johannesburg, South Africa). Because the diclofenac solution contained a small amount of ethanol, we added the same concentration of ethanol to our control saline solutions.

LPS was given intramuscularly at a dose of 100 µg/kg, a dose known to produce a marked fever in Pekin ducks (12). Diclofenac sodium was given intraperitoneally at a dose of 15 mg/kg, a dose that markedly inhibits LPS-induced fever in Pekin ducks (12).

Experimental procedures

Experiments were carried out at a regulated ambient temperature of 22 °C, which lies within the thermoneutral zone for Pekin ducks (2,27) and the study took place over a period of 5 months. Two series of experiments were done and in both series of experiments the period of restraint was from about 08:00 until 16:00 (local time) and injections were given at about 10:00. All ten birds received all six treatments in random order, and included two doses of LPS. However, because of equipment failure, two birds had no data at all, two birds did not have data for experiments done in Series I and two birds did not have
data for experiments done in Series II. Therefore, the sample size was six for each intervention.

**Series I.** The effect of prolonged restraint upon normal $T_b$ was evaluated by comparing the $T_b$ profile of the ducks in their holding stall (unrestrained) with the profile when the ducks were restrained. For restraint, the ducks were placed in a stand containing a canvas sling that comfortably supported the duck's trunk, but prevented the duck from turning around. A cotton string was tied loosely around their legs to prevent them from retracting their legs underneath their trunk. The eight hour duration of restraint was chosen because it represents a time span that covers the peak elevation in $T_b$ in response to LPS (10,11,12,17) and is a duration that has been used in previous studies (10,11,12,17). The effect of diclofenac sodium on $T_b$ during restraint was evaluated by injecting diclofenac about 2 hours after the ducks were placed in the stand. Diclofenac has no effect on the normal Tb pattern of unrestrained ducks (12) and so diclofenac treatment of unrestrained ducks was not repeated in the present study.

**Series II.** The effect of restraint upon the fever response was evaluated by comparing the $T_b$ changes induced by LPS in ducks restrained in an experimental stand, as described above, with the temperature changes induced by LPS when the ducks were in their holding stalls (unrestrained). In the latter case the ducks were removed from their stalls for LPS injection and then returned to the stalls for the remainder of the experiment. At least 14 days
elapsed between LPS injections to avoid tolerance developing to the pyrogen (9).

At the end of the experiments, the ducks were killed by an overdose of pentobarbital sodium (Euthan-Naze, Centaur Laboratories, Johannesburg, South Africa) administered intravenously according to Animal Ethics Committee guidelines, the data loggers were retrieved, and the data downloaded.

Data analysis
All data are reported as mean with standard deviations. There were no differences in baseline Tb or the responses to restraint or fever between genders and so the data from male and female ducks was pooled.

Series I: To determine whether prolonged physical restraint caused an elevation in $T_b$, and whether diclofenac sodium affected restraint-induced changes in $T_b$, a thermal response index (TRI) was calculated for each bird for each treatment. To calculate the TRIs, the change in $T_b$ from pre-restraint temperature was integrated with respect to time for the eight-hour period of restraint (or the equivalent period of time when the animals were unrestrained). The TRIs for the four treatments were compared using repeated measures ANOVA with a Tukey post hoc test. The treatments we compared were: ducks put in restraints and injected with saline [Saline (restrained)], ducks put in restraints and injected with diclofenac sodium [Diclofenac (restrained)], ducks injected with saline and returned to their stall [Saline (unrestrained)], and no intervention [Normal $T_b$]. In addition, the maximum body temperatures measured during the first two hours
of restraint (or the equivalent period of time when the animals were unrestrained) were compared using one-way ANOVA with a Tukey post hoc test.

**Series II:** To determine whether restraint affected the magnitude of the febrile response to LPS, we compared the maximum body temperatures measured during the two LPS treatments using a Student's t-test for paired comparisons. We also calculated, for each duck for each treatment in Series II, two separate 6-hour TRIs and compared them across the three treatments using ANOVA. The three treatments compared were: ducks put in restraints and injected with LPS [LPS (restrained)], ducks injected with LPS and returned to their stall [LPS (unrestrained)], and ducks injected with saline and returned to their stall [Saline (unrestrained)]. The first TRI began at the time of injection and integrated data until 6 hours after injection, when the ducks were returned to their home pens on the days of experimental restraint. The second TRI integrated data from 6 to 12 hours after injection, and so ducks were unrestrained during this period for all treatments. Because the pre-restraint T_b of the ducks did not differ between the three treatments, all TRIs were calculated as the change in temperature from pre-restraint temperature.

**Results**

**Stress hyperthermia**

The procedure of giving an injection, with or without restraint, resulted in a rapid and significant rise in T_b in the ducks compared to the values when they were left undisturbed (Fig. 1, F_{3,20} = 9.177, P < 0.01). There was no difference in the
magnitude of these increases in $T_b$ between the three treatments that involved handling the animals. When the ducks were injected with saline and returned to their stall, their $T_b$ returned within 1 hour to temperatures similar to those measured when the animals were left undisturbed. However, when the ducks remained restrained, irrespective of whether they were injected with saline or diclofenac sodium, their $T_b$ remained elevated relative to their temperatures when they were unrestrained, and did not return to the unrestrained levels until they were returned to their stall after eight hours of restraint (Fig. 1).

This sustained elevation in $T_b$ in restrained animals, even when injected with diclofenac sodium, is confirmed statistically in Fig. 2, which shows the eight-hour TRIs for each treatment. The effect of sustained physical restraint on the TRIs was significant (Fig. 2, $F_{3,20} = 9.707$, $P < 0.01$), such that the TRIs for the ducks when they were injected with saline and returned to their stall did not differ from the TRIs when they were left undisturbed, but the TRIs of the ducks when they were restrained were significantly greater than when they were unrestrained. Diclofenac had no effect on the TRI of the ducks when they were restrained.

In summary, compared to unrestrained ducks, restraint produced an elevation in $T_b$ of about 0.5°C which lasted for the duration of the restraint and was not influenced by diclofenac sodium.

_Fever_
LPS induced a fever in both restrained and unrestrained ducks (Fig. 3). The magnitude of the response, in terms of both temperature increase and duration, was greater in the unrestrained animals. The maximum febrile temperature reached after LPS injection was significantly higher when the ducks were unrestrained (43.1 ± 0.3°C) than when they were restrained (42.5 ± 0.3°C; \( t_5 = 3.053, P < 0.01 \)), despite the ducks’ \( T_b \) being higher at the time of pyrogen injection when they were restrained.

The increase in fever duration in unrestrained ducks is confirmed in Fig. 4 which shows TRIs for the first six-hour period after injection (a period of time that included the time when the birds were restrained), and TRIs for the period extending from six to twelve hours after the animals were injected (a period of time when the animals were in their stall irrespective of the initial intervention – restraint or no restraint). For the first six-hour period, LPS induced a significant rise in the \( T_b \) of the ducks compared to when they were injected with saline, irrespective of whether they were restrained after injection with LPS (Fig. 4, \( F_{2,5} = 18.90, P < 0.01 \)). There was no significant difference between the TRIs calculated for the first six-hour period following LPS injection when the animals were restrained compared to when they were unrestrained.

However, statistical analysis of the TRIs calculated for the period 6-12 hours after LPS injection showed that the fever induced when the ducks were unrestrained was maintained for significantly longer than when the animals were restrained for the first six hours of the fever (Fig. 4, \( F_{2,5} = 15.87, P < 0.01 \)). There was no difference in the TRI from 6-12 hours when the ducks were
injected with saline and when they were injected with LPS and restrained, indicating that the fever had subsided by this time when the ducks were restrained.

In summary, both restrained and unrestrained ducks developed a fever in response to an LPS injection, but unrestrained ducks reached a higher maximum febrile temperature and maintained their fever for longer.

Discussion

Although there is evidence that an increase in $T_b$ responses to emotional stimuli has an old phylogenetic origin (5,23), our understanding of the mechanisms that underlie the phenomenon are largely limited to mammals. In mammals we know that handling produces a marked rise in $T_b$ (3,4,31) and that this rise has some characteristics of a fever, resulting in the increased $T_b$ sometimes being called a “stress fever” (18). Restraint is a form of psychological stress that produces an increase in $T_b$ in mammals (24,30), as well as some of the other characteristics of fever, such as increased glucocorticoids and IL1β, and decreased food intake (16). Restraint is often used in physiological studies and until now we have not known what effect, if any, restraint has on the physiology of non-mammalian animals. Such considerations are especially important in thermoregulatory studies, since a
stress hyperthermia could alter thermoregulatory effector responses independently of any experimental manipulation that is being studied.

We have shown that in both the afebrile and febrile state, restraint had a significant effect on $T_b$ in Pekin ducks, firstly causing an elevation in $T_b$ for the duration of restraint and secondly attenuating the febrile response to a bacterial pyrogen.

*Restraint-induced increase in body temperature*
Handling of birds produces an acute increase in their $T_b$ (5,10,17,20), often by more than 1°C, which returns to pre-handling levels within 90 minutes of them being released. When handling is followed by restraint, the $T_b$ decreases over time (10,12,17), and the assumption has been, at least by some authors, including ourselves, that the $T_b$ returned to pre-restraint levels in much the same way as it does after a period of handling only. However, the present study shows that is not the case and, in fact, restrained birds maintain a $T_b$ about 0.5°C higher than unrestrained animals. That the ducks were within their thermoneutral zone is important within this context because it means that an elevation in $T_b$ could be induced by either decreased heat loss (via peripheral vasoconstriction or decreased respiratory heat loss) or increased heat production (via shivering).

Much is known about the mechanisms of psychological stress-induced increases in $T_b$ in mammals (23), revealing some similarities to fever. Acute psychological stress induces an elevation in IL1β, but not TNFα or IL6, in rats
The majority of findings support the idea that the increased temperature is a fever that occurs via both PGE$_2$-dependent and/or PGE$_2$-independent mechanisms (23,26). Because the febrile response in birds also has a PG-dependent component (1,9,12,14) we examined the effect of diclofenac sodium, an inhibitor of prostaglandin synthesis, on the response to restraint. In a previous study in Pekin ducks (12) we showed that diclofenac sodium at doses of 5 mg/kg and 15 mg/kg produced marked and equal reductions in the fever response to LPS and had no effect on T$_b$. We chose the higher dose of diclofenac sodium for the present study, confident in the likelihood that even though we didn’t measure PG concentrations, their production would be significantly inhibited. In the restrained ducks, diclofenac sodium had no effect on the induced rise in T$_b$, apparently ruling out the involvement of PGs in the elevation in T$_b$. In sharp contrast, a previous study in chickens showed that inhibition of PG synthesis with salicylate reduced the rise in core temperature induced by handling (6). There are several possible explanations for this difference. Firstly, the dose of salicylate used to inhibit PG formation causes hypothermia in that species (9) and therefore may itself have produced the reduced temperature response to handling. Secondly, the difference might lie in different mechanisms resulting in elevated T$_b$ in response to acute handling as opposed to sustained restraint, with only the former involving PGs. A third explanation is that species variations account for the difference in findings between chickens and ducks.

If the increased T$_b$ of the ducks when they were restrained was not mediated by PGs then what mediated the temperature rise? There are two likely possibilities.
Firstly, the rise in $T_b$ could still be a fever, but mediated by a PG-independent pathway. Such PG-independent pathways have previously been postulated for the pigeon (21) and the Pekin duck (12). Alternatively, the restraint-induced elevation of $T_b$ might not be a fever. Since the ambient temperature, 22°C, is within the thermoneutral zone of the ducks, the elevation in $T_b$ could be mediated by changes in either metabolic rate and/or blood flow distribution. Likely candidates for the mediation of such a response are the catecholamines, acting either centrally, as shown in mammals (18,23), or systemically. However, an involvement of peripheral vasoconstriction seems unlikely since it was excluded as a possible mechanism for the handling-induced elevation in $T_b$ seen in chickens (6).

**Restraint-induced attenuation of fever**

There have been several studies in a variety of avian species characterizing the febrile response to LPS (9,17,20,21). Evidence shows that the febrile response in birds is functionally similar to that in mammals, namely that it is mediated by cytokine-induced (9,14,21) production of PGs (1,9,12,21) and nitric oxide (12). The present study in ducks has confirmed that LPS is a potent pyrogen in birds, but has extended our understanding by showing that restraint attenuates the effects of the pyrogenic stimulus on $T_b$. Indeed, we are the first to show, in any species or phylum, that psychological stress affects the duration and magnitude of a concurrent simulated fever. The only previous study to investigate the effect of psychological stress on fever was done in rodents, and it looked at the effect of prior psychological stress, not concurrent psychological stress, on fever (13). Johnson and colleagues (13) found that a prior psychological
stressor in rats (inescapable tailshock) resulted in a higher fever and enhanced corticosterone and ACTH responses to LPS than to LPS alone. The authors suggest that the tailshock stress sensitised cytokine pathways leading to an enhanced response to the LPS stimulus. The difference may be due to the nature of the psychological stressor, prior for inescapable tailshock or contemporary for restraint, or point to differences in stress / febrile signalling between mammals and birds.

In the past, we have reported on thermoregulatory studies in both restrained (10,11,17) and unrestrained birds (12) without considering whether the psychological stress associated with restraint had any physiological consequence. Although restraint did not alter the febrile response qualitatively, it did cause quantitative changes by markedly reducing both the maximum temperature reached and the duration of the fever in response to LPS.

Although we found that the maximum febrile temperature was higher when the animals were unrestrained compared to when they were restrained, the TRIs calculated for the first six-hour period following LPS injection were not statistically different. This result suggests that restraint did not alter the first six hours of the fever. However, we believe that the initial decrease in Tb that occurred following LPS injection when the birds were unrestrained, and which did not occur when the birds were restrained, counteracted the effect of the higher febrile temperatures seen when the animals were unrestrained (Fig. 3).

The finding that stress per se influences the ducks’ response to LPS may
well be explained by an involvement of the glucocorticoid stress hormones. Glucocorticoids have been shown to modulate fever in mammals (7,8,25) and evidence supports them having a similar role in birds. For example blood concentrations of corticosterone increase during fever in birds (1, 14) and the hormone attenuates fever in Pekin ducks (12). It is therefore attractive to speculate that the corticosteroid stress hormones played a role in the inhibition of the LPS fever during restraint. Although we did not measure plasma levels of corticosterone in the present study, the likelihood that restraint superimposed an even greater increase in stress hormone response than that associated with the LPS alone is feasible. Hence, elevated blood concentrations of corticosterone in restrained as compared to unrestrained ducks may be responsible for the reduced fever magnitude and duration. Very interestingly, in mammals a psychological stress, inescapable tailshock, prior to LPS injection, resulted in both elevated corticosterone and elevated fever (13). The seemingly incompatible result can be explained by the finding that the rats exposed to the tailshock develop a resistance to the suppressive effects of glucocorticoids on cytokines and also on the HPA itself (22). It would be interesting to see if such delayed phenomena also influence fever in birds, as the attenuated fever in restrained birds indicates that they may have augmented secretion and/or sensitivity to glucocorticoids.

**Perspectives and Significance**

The findings of the present study highlight the importance of stress factors in the thermal biology, if not all physiology, of birds and presumably other experimental animals. By showing that restraint induces an increase in core
temperature and reduces the core temperature response to pyrogenic stimuli we have clearly demonstrated that restraint significantly affects normal thermoregulatory responses in birds. Our data contributes to the growing body of evidence that artificial laboratory conditions impact on the physiology of experimental animals, and laboratory-based experiments need to place experimental animals in environmental conditions as close to their natural environment as possible. This requirement holds true not only for experimental animals captured from the wild, but also captive-bred and domesticated species, such as the ducks used in our study.
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Figure 1

Body temperature (°C)

Injection

- Diclofenac (restrained)
- Saline (restrained)
- Saline (unrestrained)
- Normal

Time (hours)

-2 0 2 4 6 8 10 12

Restrained
Figure 2

Thermal response index (°C/hr)

-2 -1 0 1 2 3 4 5 6 7 8

Normal Tb  Saline  Saline  Diclofenac
Unrestrained  Restricted

Intervention

*
Figure 4

Thermal response index (°C.hr)

Saline  |  LPS unrestrained  |  LPS restrained  |  Saline  |  LPS unrestrained  |  LPS restrained

0-6 hours after injection  |  6-12 hours after injection

Intervention
References


Figure legends

Figure 1. Body temperature measured every 10-minutes in Pekin ducks (n = 6) in Series I experiments. Ducks were restrained for eight hours (indicated by black bar on abscissa from 0 – 8 hours) and injected (indicated by arrow) with Diclofenac [Diclofenac (restrained)] or Saline [Saline (restrained)], or injected with saline and released back into their home pen [Saline (unrestrained)]. The body temperature of the ducks when left undisturbed in their home pens is shown for comparison [Normal Tb].

Figure 2. The 8-hour thermal response index of Pekin ducks (n = 6) in Series I experiments. The TRI was calculated from 30 minutes before injection, which was the time that restraint was applied if required, until eight hours later, which corresponded to the time of release in restraint treatments. Stars indicate significant difference (P < 0.05) from unrestrained treatments.

Figure 3. Body temperature measured every 10-minutes in Pekin ducks (n = 6) in Series II experiments. Ducks were restrained for eight hours (indicated by black bar on abscissa from -2 to 6 hours) and injected (indicated by arrow at time zero) with LPS [LPS (restrained)], remained unrestrained in their home pen and were injected with LPS at time zero [LPS (unrestrained)], or remained unrestrained in their home pen and were injected with saline at time zero [Saline (unrestrained)].

Figure 4. The two 6-hour thermal response indices of Pekin ducks (n = 6) in Series II experiments. The first TRI (open bars) was calculated from the time of
injection, which was 2 hours after restraint was applied if required, until six hours later, which corresponded to the time of release in restraint treatments. The second TRI (closed bars) was calculated from 6 to 12 hours after injection. * indicates a significant difference from Saline 0-6 hours. † indicates a significant difference from Saline 6-12 hours. ‡ indicates a significant difference from LPS 6-12 hours.