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Empirical evidence of cold stress induced cell mediated and humoral immune response in common myna (*Sturnus tristis*)

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Abstract Common myna (*Sturnus tristis*) is a bird indigenous to the Indian subcontinent that has invaded many parts of the world. At the onset of our investigation, we hypothesized that the immunological profile of myna makes it resistant to harsh/new environmental conditions. In order to test this hypothesis, a number of 40 mynas were caught and divided into two groups, i.e., 7 and 25 °C for 14 days. To determine the effect of cold stress, cell mediated and humoral immune responses were assessed. The macrophage engulfment percentage was significantly ($P<0.05$) higher at 25 °C rather than 7 °C either co-incubated with opsonized or unopsonized sheep red blood cells (SRBC). Macrophage engulfment/cell and nitric oxide production behaved in a similar manner. However, splenic cells plaque formation, heterophil to lymphocyte (H/L) ratio, and serum IgM or IgG production remained non-significant. There was a significant increase of IgG antibody production after a second immunization by SRBC. To the best of our knowledge, these findings have never been reported in the progression of this bird's invasion in frosty areas of the world. The results revealed a strengthened humoral immune response of myna and made this bird suitable for invasion in the areas of harsh conditions.

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Introduction

It is now well known that invasive species can carry diseases into new environments (Torchin et al. 2003) and, as such, can serve as vectors, carrying new diseases to native species. This may be critical, as it may lead to the local extinction of native species (Case 1996). On the other hand, cross-species disease transmission exposes invasive species to new infectious pressure, which may limit their invasive capacity. Consequently, an enhanced immunity may increase the capacity of invading species to colonize in new territories. Moreover, invading species are likely to encounter new infectious conditions in environments that differ from their native habitat in many physical dimensions, which may increase their susceptibility to diseases. For instance, colder conditions can increase the risk of contamination in regard to several infectious agents due to the high production of corticosterones (Sandhu et al. 2013). The mynas commonly roost in winter (Khera and Kalsi 1986) and in moderate temperature (Yap et al. 2002). Common myna is a native bird from Asia that is now present in Australia and many regions of Africa. It is an unrivalled invasive bird species and has colonized many parts of the world. It is one of the world's most invasive species (IUCN) and can be a potent threat to local biodiversity and human well-being as it may be the vector of many potent parasites and local crops (Feare and Craig 1998). Because of this invasive capacity, mynas represent an excellent model to examine how the immunity of an invasive species fluctuates in relation to variable environmental conditions.

Among avian and mammalian species, the basic defense from the prevalent diseases is the body's own immune system.

Now, it is accepted that a wide range of environmental stresses have detrimental consequences on the immune system cells and their activity (Khattak et al. 2012), and extremes in ambient temperate cause elevated production of corticosterones (Lopez et al. 2007) in broiler. One cause of this immunosuppression is high levels of glucocorticoids and presence of their cytosolic receptors on B/plasma cells, hence leading to less antibody production. This induces an amplified transfer of lymphocytes from circulation to the lymphoid organs and reduced production of interleukin (IL-1 and IL-2) from macrophages (MØ) and T lymphocytes, respectively (Khattak et al. 2012). The primary defense line of a bird's body is maintained by heterophils and macrophages; these are phagocytic cells that help the immune system to get rid of apoptotic bodies, pathogens, and foreign material (Qureshi et al. 1986; Sandhu et al., 2013). In MØ, these engulfed materials are processed with lysozymes for denaturation and killing mechanism of microorganisms by oxide radical production (Sandhu et al. 2007, 2012, 2013) and one of them is nitric oxide. Heterophil to lymphocyte ratio is frequently used to assess avian welfare (Nicol et al. 2006). Moreover, this ratio can be used to assess future susceptibility of diseases in newly hatched chicken (Sandhu et al. 2013). As the myna has been introduced to many parts of the world (deliberately or through invasion), it is important to look into the immunological profiles of the myna to combat the diseases in harsh environmental conditions. The widespread distribution of myna from subcontinent to Middle East, Australia, New Zealand, and South Africa has made it an important invasive species to survive in wide ranges of temperatures.

Materials and methods

Animal husbandry and experimental design

The present study was carried out on adult and sexually mature myna. A number of 40 adult (110–120 g live body weight), wild myna were caught and morphologically identified by a zoologist, PMAS-Arid Agriculture University Rawalpindi. The birds were maintained in an environmentally controlled house with 16:8 h light (16 h light period and 8 h dark). At the time of trapping, the ambient temperature in the area was 30.9 °C in the morning and 21 °C at the evening. Upon arrival, the birds were randomly subdivided into two different groups having 20 birds each and were acclimatized at a temperature of 25 °C for a period of 10 days and then exposed to two temperature regimes (7 °C±2, 25 °C±2) for 14 days. Before the start of treatment, the birds were additionally subdivided into four replicates, having five birds each. All the experimental protocols were followed by the permission of the Institutional Advanced Study Research Board which is the main University Ethics Committee.

Abdominal exudate cell collection

After 14 days of temperature treatment, three birds from each replicate were decapitated for a surgical exposure of the abdominal cavity to collect abdominal exudate cells (AEC). The procedure of AEC collection was already standardized in our laboratory as described elsewhere (Sandhu et al. 2012). Briefly, a 3 % pre-swollen suspension of sephadex G-50 wt/vol granules (Sigma Chemical Co., St. Louis, MO) in normal saline was injected intraperitoneally at the rate of 1 ml/100 g body weight about 42 h before AEC collection. A 20-ml solution of chilled normal saline and heparin (0.5 U/ml) was intraperitoneally injected, and the peritoneal cavity was massaged to disentangle resident MØ. The collected AEC suspension was poured into seliconized tubes kept on ice for 20 min to settle down debris and left over sephadex granules and then was centrifuged at 1,500 rpm for 10 min at 4 °C. The cell pellet was re-suspended in Rosewell Park Memorial Institute (RPMI-1640) cell culture media and washed to acquire pure MØ culture.

Culture of MØ

The obtained cells were checked for their morphology and viability with trypan blue dye. The MØ viability was found 97 % and their final concentration of 2.9×10^4/20 μl was adjusted. The cells in 20 μl suspension were cultured on sterilized round glass cover slip (18 mm) for 30 min to get MØs attachment. The whole process was carried out in fresh RPMI-1640 with antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin) supplemented with 5 % fetal bovine calf serum at 39.6 °C in 5 % humidified CO_2 chamber for cellular studies.

Preparation of hyper immune sera and opsonized SRBC

The hyper immune serum against sheep red blood cells (SRBC) was prepared in three commercial broiler chickens as described by Sandhu et al. (2007). On day 1, 0.5 ml of washed 2.5 % SRBC suspension in phosphate buffer saline (PBS) and equal volume of Alsever's solution (Sigma Chemical Co., St. Louis, MO) was injected subcutaneously under the wing vein of three broiler birds. After 7 days, the injection was duplicated and re-injected after 15 days of the first inoculation. Following the first inoculation, blood was collected from jugular vein for separation of serum that was preserved in polystyrene sterile tubes for further use. Serum anti-SRBC antibodies were observed by hemagglutination assay, and opsonization of SRBC was carried out as described already by Sandhu et al. (2006). For opsonization, SRBC were incubated with sub-agglutination concentration of heat-inactivated anti-SRBC antibodies overnight at 4 °C and their final concentration of 2.5 % was maintained in RPMI-1640.

Unopsonized SRBCs solution of 2.5 % was prepared separately in RPMI-1640 medium.

In vitro cytotoxicity activity of macrophages

The whole process of in vitro cytotoxic activities was carried out in the Petri dishes containing four cover slips (Sandhu et al. 2006) each. Briefly, post 30-min incubation in cell culture media (at 39.6 °C with 5 % CO_2 and 95 % fresh air), all the cover slips with MØ were washed and transferred to the new medium. For engulfment capability of these adherents, MØ was dogged by 2.5 % unopsonized and opsonized SRBC suspension and was incubated at 39.6 °C in 5 % CO_2 and 95 % fresh air for further 2 h. Cover slips were removed and washed with normal saline for exclusion of any free SRBC, fixed in methanol for 10 min, stained with May-Grünwald-Giemsa (Merck, Chemical Co., Darmstadt, Germany), and were mounted on clean glass slides with DPX. A number of 200 cells/cover slip were counted for engulfing percentage which was calculated as follows:

$$\text{Engulfment Percentage} = \frac{\text{Total cells engulfing in an area}}{\text{Total cells present in an area}} \times 100$$

For opsonization, antibodies against sheep RBC were developed in broiler birds as we have already stated (Sandhu et al. 2006) and these polyclonal antibodies were monitored by hemagglutination assay using 2.5 % SRBCs and the highest titer of 1:64 was obtained. For SRBC opsonization, equal part of heat-inactivated antiserum (diluted to 1:128) was mixed with 2.5 % SRBC and kept for 2 h at 4 °C.

Nitrite determination

The cultured MØ were stimulated with different antigens and bacterial lipopolysaccharides (LPS) for the determination of nitrites in the culture medium. The nitrites in term of nitric oxide were detected with the reaction of Griess reagent (Green et al. 1982; Sandhu et al. 2013). Briefly, 1 ml of MØ culture supernatant was transferred to the 96-well flat bottom microtitration plates, combined with Griess reagent [1 % sulfanilamide (Sigma Chemical Co., St. Louis, MO) and 1 % naphthylethylenediamine dihydrochloride (Merck, Chemical Co., Darmstadt, Germany)], and incubated at room temperature for 15 min. Nitrite presence was indicated by reading the plates at A_{492} on an ELISA plate reader (BioTEK, ELX-800©). Nitrite concentration was calculated by sodium nitrite (10–100 mM) standard curve.

Concentration of *Sturnus tristis* serum IgG and IgM

The birds were injected with 1 ml of 2.5 % SRBC suspension 7 days prior to blood collection. After 7 days, the 2nd shot of same dose was injected in the birds after collection of wing vein blood. The blood was drawn again after 14 days of first injection and serum was recovered. The concentration of serum IgG and IgM was determined by 2-mercaptoethanol (2-ME) sensitivity assay. Mercaptoethanol (Fluka Chemical Co., West St. Paul) is used to seize binding capability of IgM antibody, while IgG affinity remained unaffected.

Plaque forming cell response of *S. tristis* splenic cells

The plaque formation by splenic cells was enumerated by using SRBC modified hemolytic plaque assay (Jerne and Nordin 1963). Briefly, the birds were immunized with 2.5 % washed SRBC (0.2 ml/myna) by intravenous injection. After 4 days, splenocyte suspension was geared up by crumbling the spleen between two frosted slides, washed the slides with RPMI-1640, and whole suspension was returned into test tube of 5 ml for centrifugation at 300×*g* for 10 min. The plaque forming cell (PFC) assay was performed as previously described by Anderson et al. (2007). The results of PFC were expressed and recorded as the number of IgM-PFC per spleen.

Heterophil to lymphocyte ratio (H/L)

The glass slide smears were made, dried, fixed in methanol, and stained with May-Grünwald-Giemsa stain. The H/L ratio was determined by counting 200 cells from each slide at ×1,000. The cells were recognized on the basis of their morphology. All of the H/L counts were carried out by the same researcher.

Concentration of circulating corticosterones

At the time of decapitation, 1 ml of blood was collected in the vacutainers coated with gel for the separation of blood serum. The separated serum was stored at −4 °C for further evaluation of blood corticosterones (ng/ml) through ELISA using a kit (DRG Diagnostics, Marburg, Germany, Ref. No. EIA-1887).

Data analysis

All of the data from different categories was collected and analyzed statistically using completely randomized design (SAS Institute). The data was subjected to ANOVA and is presented as means±SEM. If there was a significant variation between means, the data was subject to the Duncan Multiple Range (DMR) test to find out the significance of data under consideration. The $\log^2$ conversion of total Ab, IgG, and IgM

titers was carried out before statistical analysis. The significance level was set at $P<0.05$.

Results

Innate immune response

Macrophages derived from the myna kept at 25 °C significantly phagocytosed ($F=21.904$; $P<0.001$) the opsonized SRBC as compared to 7 °C, however remained non-significant when con-incubated with unopsonized SRBC at both environmental temperatures (Fig. 1a). Similarly, MØ engulfment/cell was kept elevated ($F=6.899$; $P<0.05$) at 25 °C after opsonized SRBC co-incubation and was non-significant during unopsonized SRBC treatment irrespective of environmental temperature (Fig. 1b). Overall macrophage NO production was greater ($F=154.218$; $P<0.001$) when incubated with bacterial LPS at 25 °C and least when there was no stimulus to the MØ and at 7 °C even with opsonized SRBC stimulation (Fig. 1c). The single cell antibody production (plaque forming units/spleen) resulted in a non-significant effect of environmental temperature on formation on plaques (Fig. 2a).

Humoral immunity

Heterophil and lymphocytes play a significant role in the engulfment of pathogens and production of antibodies. There was no effect of environmental temperature on the blood H/L ratio as described in Fig. 2b. To find out the production of anti-SRBC antibody during 7 and 25 °C, we injected washed SRBC through intravenous route. The results represent that there was a significant increase in the production of anti-SRBC antibodies after 7 days of temperature treatment, while the cold temperature stress did not induce any significant influence on production of antibodies. However, there was a significant boost ($F=14.258$; $P<0.05$) in the total Ab production against SRBC after 14 days of the first I/V injection (Fig. 3a). Similarly, the decrease was significant ($F=14.315$; $P<0.05$) in the production of IgM subtype of Ab and significant increase ($F=154.218$; $P<0.05$) in the production of IgG (Fig. 3b, c) after 14 days of first I/V injection of SRBC.

Production of corticosterone

As shown in Fig. 4, cold stress has enhanced the production of corticosterone. Serum corticosterone production remained

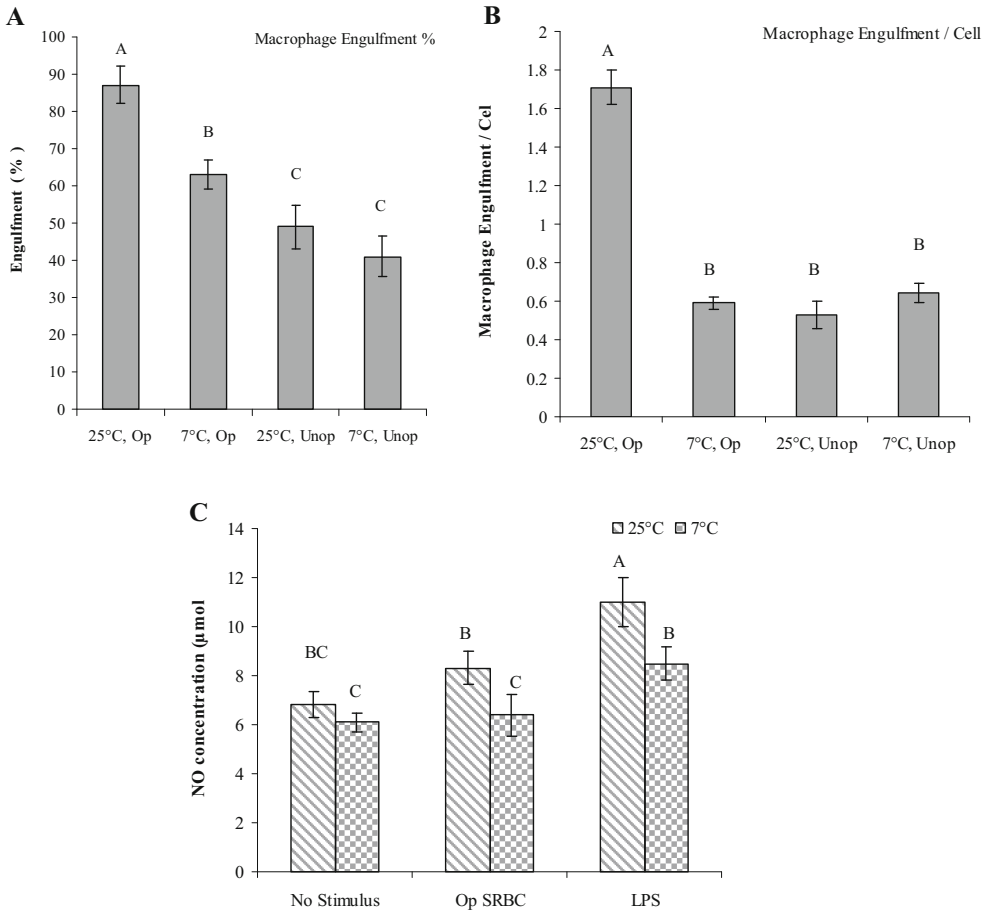

Fig. 1 Macrophage cytotoxic activity and nitric oxide production of common myna at 25 and 7 °C. **a** Macrophage engulfment percentage; **b** macrophage engulfment/cell; **c** macrophage nitric oxide production. **a**–**c** Similar alphabets do not differ significantly. All the data has been presented in mean± SE

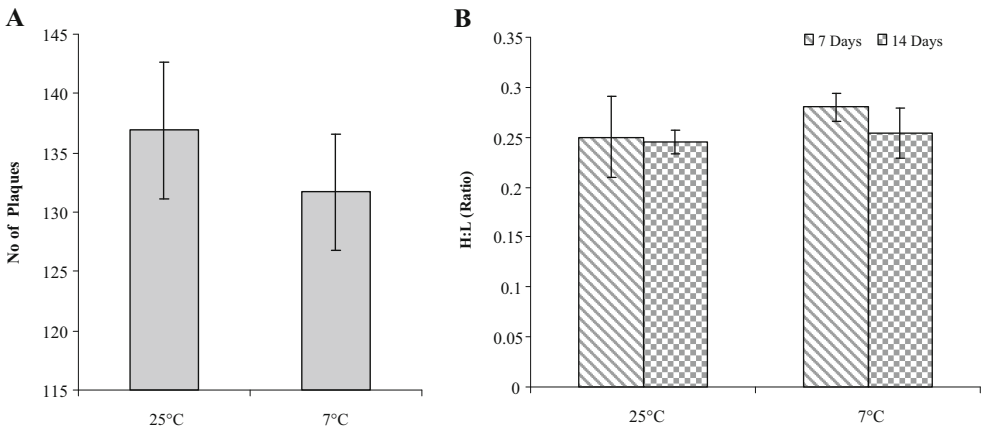

Fig. 2 Effects of cold stress on splenocyte plaque formation and heterophil to lymphocyte (H/L) ratio in common myna. **a** Number of splenocyte plaque formation; **b** heterophil to lymphocyte ratio. The data given is standard error (SE) of the mean

significantly high (F=6.700; P<0.05) at 7 and 14 days of cold stressed myna rather than birds kept at 25 °C.

Discussion

In this experiment, we kept the myna at 7 °C, according to the lowest per month temperature records of winter season in the Potohar regions of Pakistan which prevailed during last 30 years (1981–2013; Pakistan Meteorology Department). After invasion in an area, the myna has to survive in new environmental conditions those may either be favorable or harsh. These birds are found in a wide range of environmental conditions after invasion from 23.2 to −0.4 °C in Australia (Martin 1996), in Ankara, Turkey (Bilgin 1996), and in South Africa (Peacock et al. 2007) as compared to their natural South Asian temperature zone. This is possibly the first observation on the immunological profile of myna under cold environmental temperatures. Our study has validated and effectively showed the immunological changes in innate and humoral immune response of Indian myna when kept at low environmental temperature. Avian species lack resident peritoneal MØ, and therefore we attempted to mobilize abdominal MØ through an intra-abdominal injection of Sephadex G-50 (Sandhu et al. 2007, 2013) similarly as in chicken. There was a significant increase in the MØ engulfment percent and engulfment/cell at 25 °C as compared to 7 °C. This increasing trend is in accordance to the findings of (Powell 1987; Van-Loon et al. 2004) that avian MØ have more tendencies to engulf opsonized particles rather than un-opsonized Ag. Another reason of increased phagocytosis may be due to the fact that phagocytosis is facilitated by antibody-mediated opsonization and the presence of Fc receptors on these MØ (Guilliams et al. 2014). The activation of Fc receptor strengthens the phagocytic ability, processing of opsonized antigens (Yamamoto and Johnston 1984), and formation of SRBC rosette around MØ. However, low numbers of engulfing MØ were observed in birds exposed to 7 °C temperature. This may be due to decrease in phagocytic receptors, inactivation or reduction in the membrane transport/diffusion due to cold stress. However, in mice, MØ are more phagocytic after cold stress due to activation of Fcγ, mannose, and β-glucagon receptors (Baccan et al. 2010). During stress, stimulation of β-adrenergic receptors suppresses the cytokines and chemokines production by MØ and ultimately inhibition of IFN-γ induced MØ activation (Karp 2012) and involve hypothalamic-pituitary-adrenal axis. This may result in an increased risk of morbidity and mortality due to infection (Sandhu et al. 2006, 2007, 2012).

Macrophage NO production was less when stimulated with SRBC than LPS, and this may be due to decreased engulfment or Fc receptor non-stimulation. The presence of Fc receptors helps to build up lysosomal hydrolysis method through reactive oxygen intermediates (ROI) production against the engulfed Ag (Yamamoto and Johnston 1984). It is noteworthy that type I IFN does not provoke NO synthesis; nevertheless, chicken INF-δ influences its synthesis (Digby and Lowenthal 1995). In the body, infectious agent's vulnerability influences the change in macrophage ROI production (Tasat et al. 2003). Our laboratory work has previously reported similar results and correlation of LPS with NO production (Sandhu et al. 2007, 2012). A publication by Nguyen et al. (2011) suggests that cold stress up-regulates the other activation processes of tissue macrophages and probably decrease/stop inflammatory responses. The results of H/L ratio represent no effect of treatment and duration of stress. The results of the present study are dissimilar to Rajkumar et al. (2011) as they reported the increase in H/L ratio of naked neck chicken during cold stress. This shows that with the decrease in environmental temperature (7 °C), there is no/less symptom of

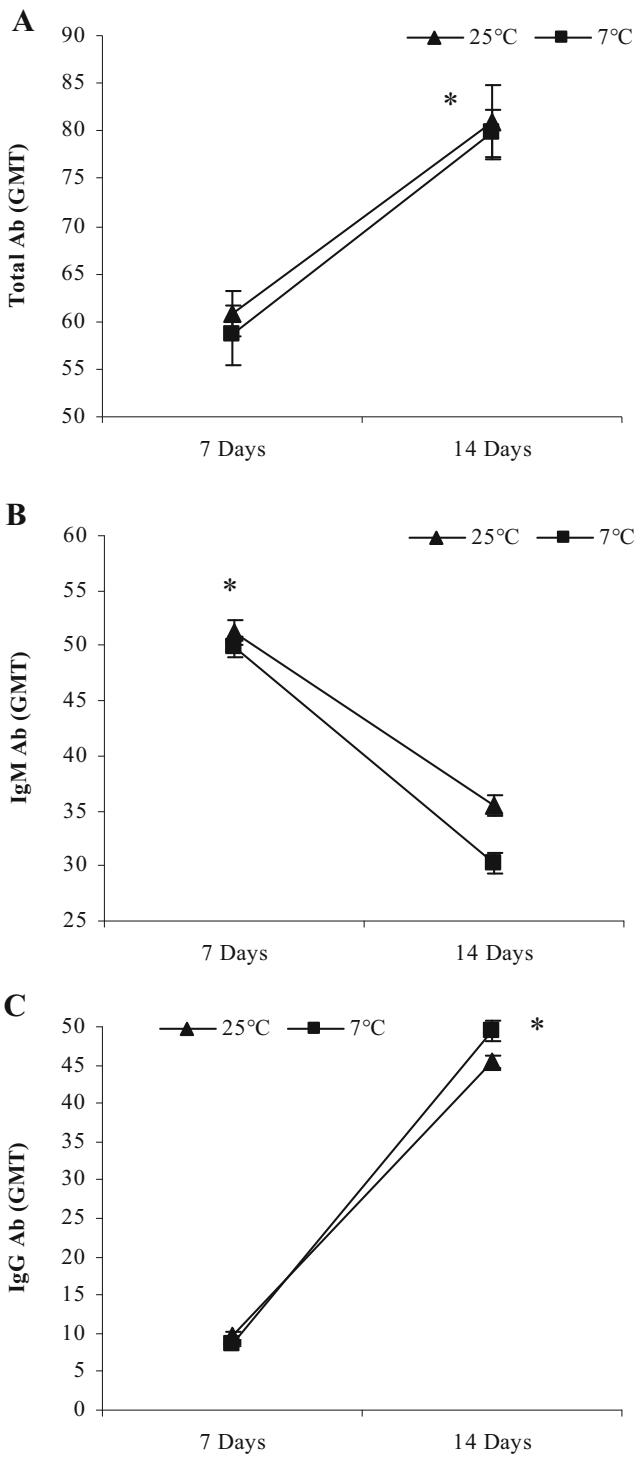

Fig. 3 The swing of cold stress against SRBC and the subtypes of antibody immunoglobulin titers. Data are mean±SE of $\log^2$ total antibody titers against SRBC; **a** the total antibody production against SRBC; **b** IgM antibody titers to SRBC; **c** IgG antibody subtype production against SRBC. *$P \leq 0.05$ indicates significant difference

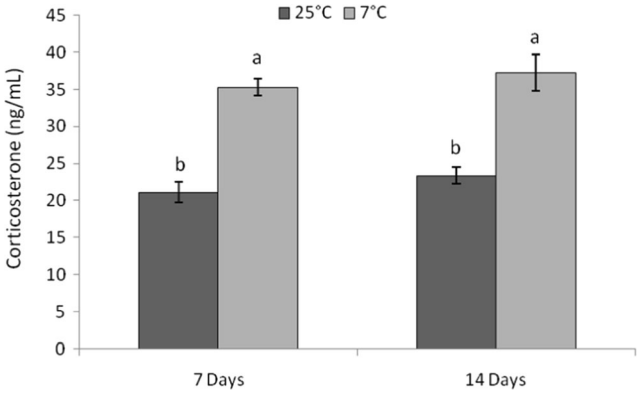

Fig. 4 The production of serum corticosterone (ng/ml; mean±SE) after 7 and 14 days of cold treatment. *a* and *b*=values with different alphabets differ ($P \leq 0.05$)

stress in myna than chicken, which makes it more suitable to invade in different cold regions of the world. To find out the effects of decreased environmental temperature on T cell-dependent immune function, an in vivo cautious measure of the host defense response was determined. For this, birds were challenged with SRBC antigens to evaluate the proportion of responding splenocytes. Our results indicate no differences in the change of environmental temperature on T cell-mediated response in myna. Among the chicken lymphoid organs, majority of circulating T cell subpopulation TcR^+ (γ/δ) lymphocytes are $CD8^+$ (Sowder et al. 1988) and ideally located in the spleen and intestinal epithelium. This can suggest that the proliferation of receptors and TcR^+ cells is same under different temperature conditions; hence, no suppressor activity of T cells or their receptors was observed during temperature variation. On the other hand, immunoglobulins are represented on B cells and thus commence activation, differentiation, and apoptosis induction (Hase et al. 2002) in the Ag. This consequently plays a vital role in the mediation of humoral immunity. According to our results, there was an abrupt increase in total Ab against SRBC from 7 to 14 days after the 2nd injection of SRBC. This is a normal physiological response that after 7 days the subtype of antibodies production switches from IgM towards the IgG type of antibodies (Sandhu et al. 2007). These results are in correlation with Dabbert et al. (1997) who found no change in humoral immunity after cold stress in *Colinus virginianus* and in laying hens. In both chicken and wild birds, the impact of cold stress on cellular or humoral immune responses is not consistent as Svensson et al. (1998) and Regnier et al. (1980) reported the decrease of humoral immunity in blue tits and in laying hens, respectively. Our study concludes that myna can acclimatize with the environmental temperature changes since there was no change in T cell-induced immunity and humoral immunity. The worldwide dispersion of myna may be due to the fact that they may perform better against different pathogens even under stressful environmental conditions.

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