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Effect of the Migratory Flight on the Immune Functions of Male Black-Headed Gulls *Larus ridibundus*

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Abstract: The long migratory flights yield to stressful physiological conditions for birds. We have addressed the study of the immune response of splenic and thymic mononuclear leukocytes from black-headed male gulls *Larus ridibundus*, searching for a putative effect of the migration cycle on the function of these leukocytes. The immune response was studied after the migratory flight (post-migratory period) and before they start the flight (premigratory period) preceded for a long resting period. The functions assayed were: chemotaxis, lymphoproliferative response to mitogens and natural killer (NK)-like cell-mediated cytotoxicity. Experimental groups were constituted on the basis of the age of the animals, because this parameter is considered to induce different patterns of immune response. We have found that the immune response of the mononuclear leukocytes is affected by the migratory cycle. An immunosuppression was observed in the post-migratory period in chemotactic mobility and even in the response to Con A and LPS of splenic mononuclear leukocytes. Different patterns were observed in the other responses studied. Our results suggest the existence of different sensitivity of the leukocyte subpopulations to the effect of the migratory flight in gulls. Abbreviations: Con A, concanavalin A; cpm, counts per min; FBS, fetal bovine serum; FMLP, N-formyl-Met-Leu-Phe; NK, natural killer; LPS, lipopolysaccharide; PBS, phosphate-buffered saline solution; PHA, phytohaemagglutinin; PWM, pokeweed mitogen.

Key words: Gull, spleen, thymus, mononuclear leukocyte, migratory cycle, chemotaxis, mitogens, NK

Introduction

Birds have an immune system with a well-developed lymphoid tissue including a hematopoyetic bone marrow, thymus, bursa of Fabricius, lymphatic nodes and lymphoid tissues associated to different locations (Le Dourain *et al.*, 1980 and Sharma, 1997). The precursors of lymphoid cells migrate from the bone marrow to the different lymphoid tissues where they mature, mainly thymus for T lymphocyte (Le Dourain *et al.*, 1977) and bursa of Fabricius in the early ages and spleen later for B lymphocytes (Jankovic *et al.*, 1979). Once they have matured they start to recirculate in order to maintain the immune vigilance (Selvaraj and Pitchappan, 1988). Mature bird leukocytes share similar surface markers than those of mammals (Thomas, 1989; Fowlkes and Pardoll, 1989; Corbel and Thomas, 1990; Lillehoj and Chung, 1992). The immunocompetence of bird leukocytes have been widely studied in poultry (Higgins, 1991; Sharma and Belzer, 1992 and Kaspers *et al.*, 1993).

On the other hand, seasonal variations affecting to the immune function has been described in poikilotherms (Saad *et al.*, 1987 and Muñoz *et al.*, 2000) and changes in the immune function have been reported associated to the reproductive period in birds (Rodríguez and Lea, 1994). Considering migratory birds, whose physiology is strongly affected by the migratory cycle that induces deep changes in these animals (Guglielmo *et al.*, 2001 and Battley *et al.*, 2001), the immune response could be also modified by the long migratory flight, since the immune system is very sensitive to stress (Sato and Glick, 1970). Indeed we have reported that splenic leukocytes from female black-headed gulls have a lower chemotactic mobility and proliferative response after the migratory flight (Muñoz and De la Fuente, 2003).

In the present work we have addressed the study of the immune function of mononuclear leukocytes from the major lymphoid organs, spleen and thymus, in migratory male black-headed gulls. The functions studied were those most representative to demonstrate the immunocompetence of leukocytes *in vitro*: mobility directed to a chemoattractant gradient (chemotaxis), lymphoproliferative response to mitogens and natural killer (NK)-like cell-mediated cytotoxicity. The study was performed after the migratory flight (post-migratory period), which could yield an immunodepletion and before they start the annual migratory flight (premigratory period) having a previous long resting period. Furthermore, we looked for different patterns of response depending on the age of the gulls (juveniles versus adults), since age has been described to modify the immune response (Ferrández and De la Fuente, 1999).

Materials and Methods

RPMI 1640 enriched with L-glutamine and HEPES, fetal bovine serum (FBS), gentamicin and pokeweed mitogen (PWM) were purchased from Gibco (Burlington, Ontario, Canada); Ficoll-Hypaque, lipopolysaccharide (LPS) from *Escherichia coli*, N-formyl-Met-Leu-Phe (FMLP) and scintillation liquid from Sigma (St Louis, MO, USA); phytohaemagglutinin (PHA) and concanavalin A (Con A) from Flow Laboratories (McLean, VA, USA); transparent chemotaxis filters from Millipore (Bedford, MA, USA); 96-well flat-bottomed and 96-well U-bottomed microtiter plates from Costar, (Cambridge, MA, USA); [^{3}H]-thymidyne (81 Ci mmol^{-1}) from Dupont (Boston, MA, USA); ^{51}Cr (1 mCi ml^{-1}) from New England Nuclear (Boston, MA, USA); harvest filter paper from Whatman Ltd. (Maidstone, UK); Phosphate-buffered saline solution (PBS) was prepared as follows: 123 mM NaCl, 11 mM Na_2HPO_4 and 3 mM KH_2PO_4. Phenol red-free Hank's solution was 5.5 mM glucose, 1 mM $MgCl_2$, 136 mM NaCl, 5 mM KCl, 1mM $CaCl_2$, 0.8 mM $MgHPO_4$, 0.5 mM KH_2PO_4, 0.4 mM Na_2HPO_4 and 4 mM $NaHCO_3$, adjusted to 7.4 pH.

Animals: The black-headed gull *Larus ridibundus L., Laridae* is a migratory species that arrives in Spain during autumn and spends the winter there. At the end of this season the gulls migrate to northern Europe, returning to Spain in the following autumn. In the present study, male gulls were captured in the Spanish countryside, with legal permission from the Autonomous Government of Madrid (Spain). Sampling was performed just once per season. Thus, sampling in the post-migratory period was performed on the 1 November (when gulls had just arrived); and, in the premigratory period on the 3 March (when the gulls start to migrate). The size of the groups (juveniles and adults) was determined by the random capture technique. Juvenile gulls (1-2 years old) were identified by the different colors of the feathers in regard to adults (Bermejo *et al.*, 1986). Animals were anaesthetized with chloroform and decapitated according to the guidelines of the European Union Council Directives 86/6091 E.U.

Splenic Mononuclear Leukocyte Isolation: Spleens were removed aseptically and stored at 4°C. They were taken to the laboratory and then minced with scissors and gently pressed on a mesh screen to obtain a cell suspension in RPMI 1640 medium plus 10% heat-inactivated FBS and 100 μg ml^{-1} gentamicin. The cell suspension was centrifuged in a Ficoll-Hipaque gradient with a density of 1.080. The cells were washed three times in PBS. The splenic mononuclear leukocytes obtained consisted of lymphocytes plus approximately 9% of monocytes, as was assessed by the cytocentrifugation of splenic cell suspensions. Cell viability was evaluated by the trypan blue exclusion test showing a viability higher than 96%.

Thymyc Mononuclear Leukocyte Isolation: Thymi were removed aseptically and stored at 4°C. They were taken to the laboratory and freed of fat, minced with scissors and gently pressed on a mesh screen to obtain a cell suspension in RPMI 1640 medium plus 10% FBS and 100 μg/ml gentamicin. The cell suspension was washed three times in PBS. The thymic mononuclear leukocytes obtained consisted of lymphocytes plus approximately 3% of monocytes, as was assessed by the cytocentrifugation of thymic cell suspensions. Cell viability was measured by using the trypan blue exclusion test showing a viability higher than 97%.

Chemotaxis Assay: The chemotaxis assay was carried out following a modification (Muñoz *et al.*, 2000) of the original technique described by Boyden (1962), which basically consists in the use of chambers with two compartments separated by a nitrocellulose filter with a pore diameter of 3 μm; 300 μl aliquots of the leukocyte suspension (5 x 10^5 cells ml^{-1} in Hank's solution) were placed in the upper compartments of the chambers. Aliquots of the well-known chemoattractant agent for leukocytes FMLP at 10^{-8} M (Schubert and Müller, 1989) were placed in the lower compartments. For the controls, chemoattractant-free medium was used. The chambers were incubated for 3 h at 37°C. Filters were then fixed and stained and cells migrating through the filter were counted in one-third of the lower face of the filter. Chemotactic indexes were calculated giving to the control a 100% value.

Lymphoproliferative Response to Mitogens: Leukocytes at a concentration of 2 x 10^5 cells/200 μl well^{-1} were seeded in 96-well plates in RPMI 1640 medium plus 10% FBS and 100 μg ml^{-1} gentamicin; 20 μl of mitogens or Hank's solution (controls) was also added to the wells. Mitogens used in this assay were 10 μg ml^{-1} PHA, 1 μg ml^{-1} Con A, 12,5 μg ml^{-1} LPS and 1:150 dilution of PWM. Cells were maintained for 72 h in an incubator at 37°C and humidified atmosphere with 5% CO_2 and 0.5 μCi of [^{3}H]-thymidine/well were added 24 h prior to the collection of cells. Cells were harvested and [^{3}H]-thymidine uptake was measured in a Beckman LS 6000 scintillation counter (Palo Alto, CA, USA). Results were expressed as the percentage of [^{3}H]-thymidine uptake (cpm; counts per min), assuming a control uptake of 100%.

NK-like Cell Mediated Cytotoxicity Assays: The target cells used in the cytotoxicity assays were the human

myeloid erythroleukemia cell line K-562. These assays were performed following the method previously described by Muñoz *et al.* (2000). Briefly, target cells, maintained in RPMI 1640 medium, were labeled separately with ^{51}Cr (100 μCi/5 x 10^6 cells) for 90-120 min at 37°C, shaking every 15 min and adjusted to 2.5 x 10^4 cells/ml. Splenic and thymic mononuclear leukocytes (effector cells) were adjusted to 2.5 x 10^6 cells/ml and added at 25:1 (effector : target cell ratio) for cytotoxicity assays.

U-bottomed microtiter plates with target and effector cells were incubated for 4 h at 37°C and then centrifuged for 10 min at 400 g. Aliquots of 100 μl were taken from the supernatants and counted in a gamma-counter (LKB, Uppsala, Sweden). The cytotoxicity percentage was calculated as follows: Cytotoxicity (%) = [(L - S) x 100] / (T - L), where L is the lysis measured by cpm released in the presence of effector cells; S is the mean of cpm spontaneously released by target cells incubated with medium alone; and T is the mean of total cpm released after incubating the target cells with 1:100 dilution of Triton X-100.

Statistical Analysis: Data are expressed as the mean ± SEM of the values from the number of experiments performed in duplicate or triplicate as indicated in the corresponding tables and figures. Data were evaluated statistically using the one-way ANOVA by addition of Kruskal-Wallis ranges for multiple comparisons, the minimum significance level being $p < 0.05$.

Results

Chemotaxis: Splenic and thymic mononuclear leukocytes from juvenile and adult gulls *L. ridibundus* were induced to migrate by FMLP (Table 1). The lowest chemotaxis indexes were obtained in the post-migratory period for mononuclear leukocytes from both spleen and thymus compared to the results obtained in the premigratory period. Differences in the mononuclear leukocyte chemotaxis indexes in relation to the age of the animals were not found.

Proliferative Response: The mitogen-induced lymphoproliferative responses of gull splenic mononuclear leukocytes are shown as percentages in Fig. 1. With the mitogen PHA (Fig.1A), the highest proliferation percentages were found in the post-migratory period for juvenile gulls compared to the results in the premigratory period. Mononuclear leukocytes from adults did not show changes in the response to PHA associated to the migratory period (Fig. 1A). Con A induced higher proliferative percentages in the premigratory period than in the post-migratory period for mononuclear leukocytes obtained from both juvenile and adult gulls (Fig.1B). Con A yielded the highest proliferation percentages observed in this study. LPS (Fig. 1C) also induced the highest proliferative action on the splenic mononuclear leukocytes in the premigratory period, but just for juvenile gulls. The proliferative response to PWM (Fig. 1D) reached the highest values in the post-migratory period for juvenile gulls. Mononuclear leukocytes from adults did not show any migratory-associated variation in the response to PWM and it was also obtained with PHA and LPS.

In regard to the effect of age on the splenic mononuclear leukocyte proliferative response, values from juvenile gulls were higher than those from adults with PHA in the post-migratory period (Fig. 1A) and with Con A in the premigratory period (Fig. 1B). There were not more differences in the mitogenic response attending to the age of the gulls for splenic mononuclear leukocytes.

The lymphoproliferative responses of gull thymic mononuclear leukocytes are shown in Fig. 2. The mitogen PHA (Fig.2A) induced the highest proliferation percentages in the post-migratory period for adult gulls compared to the results in the premigratory period. Mononuclear leukocytes from juveniles did not show changes in the response to PHA associated to the migratory period (Fig. 2A). With Con A (Fig.2B), higher proliferative percentages were obtained in the post-migratory period than in the premigratory period for mononuclear leukocytes obtained from juvenile gulls, while there were not differences in the response regarding adults. LPS and PWM were not assayed in thymic mononuclear leukocytes since these are mitogenic for B lymphocytes, which are reported to be scarcely in thymus (Butcher, 1986).

Age-associated differences in the proliferative response from thymic mononuclear leukocytes were observed with PHA in the post-migratory period (Fig. 2A), when the percentages obtained with cells from adults were higher than those from juveniles. With Con A (Fig. 2B) the highest response was obtained in thymic mononuclear leukocytes isolated from juveniles in the post-migratory period. There were not differences in the response attending to the age of the gulls during the premigratory period (Fig.2).

NK-like Cell-mediated Cytotoxicity: The effect of the migratory cycle on the NK-like cell-mediated cytotoxicity was evident in splenic mononuclear leukocytes (Fig. 3A). These cells, from both juvenile and adult gulls, showed the highest cytotoxic activity in the post-migratory period. There were not differences in the cytotoxic activity of thymic mononuclear leukocytes (Fig. 3B) regarding the migratory period.

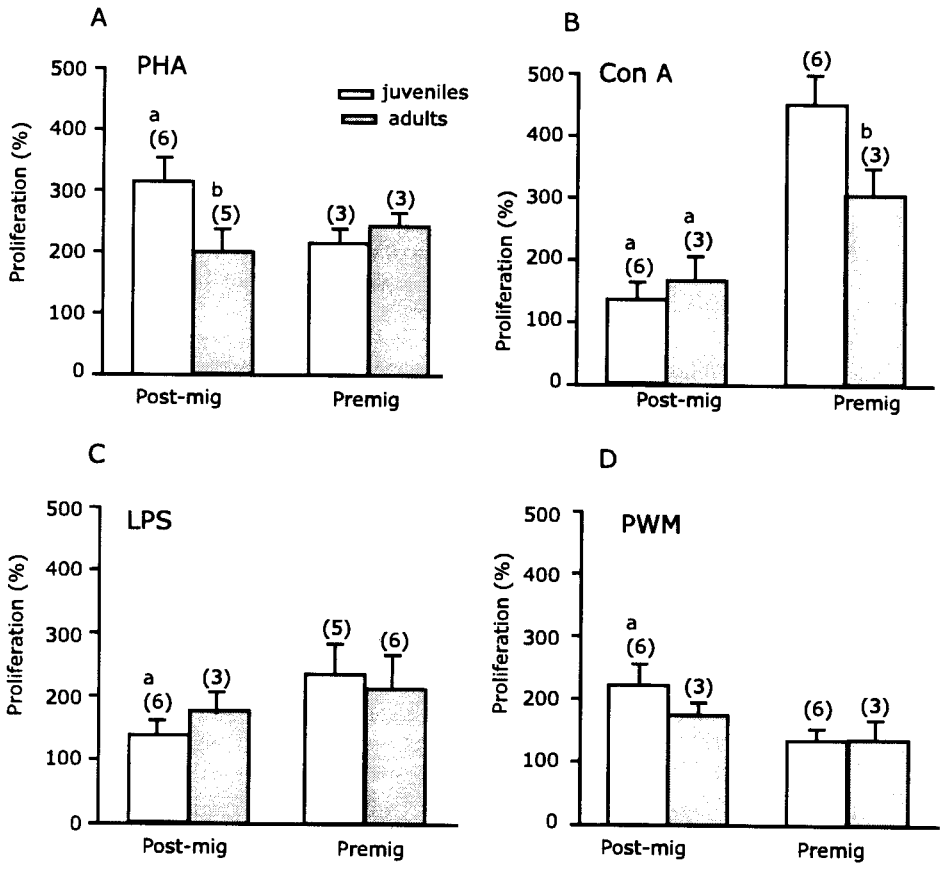

Fig. 1: Effect of the seasonal migratory cycle on the proliferative response of splenic mononuclear leukocytes to the mitogens PHA (A), Con A (B), LPS (C) and PWM (D). The results are the mean ± SEM of the number of animals (in parentheses) on which the determinations were performed in triplicate. a $P<0.05$ versus premigratory; b $P<0.05$ vs. juveniles

Splenic mononuclear leukocytes from juvenile gulls showed a higher NK-activity against K-562 cells than those from adults in the premigratory period (Fig. 3A). No age-associated differences were observed in the cytotoxic activity of thymic mononuclear leukocytes.

Discussion

We have studied the most representative leukocyte functions, i.e. chemotaxis, proliferation and NK-like cell-mediated cytotoxicity, in gull splenic and thymic mononuclear leukocytes addressing the effect of the long migratory flight on the immune response.

Mononuclear Leukocyte Functions: We have found that both splenic and thymic mononuclear leukocytes from males of *L. ridibundus* have chemotactic-directed mobility, as reported for splenic leukocytes from females of the same species (Muñoz and De la Fuente, 2003) and both sexes in chicken (Joshi and Glick, 1990). Since chemoattractant-directed mobility is a key characteristic of the immune vigilance that guarantees the effectiveness of the immune response (Damle and Doyle, 1990), we could suggest that the immune migration to the infective focus in gulls involves similar mechanisms to those of mammals.

The mitogen-induced proliferation is a experimental assay to measure the capacity of response to antigens (Nowell, 1960 and Andersson *et al.*, 1972). Splenic and thymic mononuclear leukocytes from *L. ridibundus* present a positive response to all the mitogens assayed, thus the antigen recognizing systems could share similar mechanisms with those of mammals. Similar results have been reported for chickens (Holt, 1990) and ducks (Higgins, 1991).

NK-mediated cytotoxicity is used by a leukocyte subpopulation to eliminate different targets as tumoral cells, fungi, bacteria and parasites (Miller, 2001). Splenic and thymic mononuclear leukocytes from *L.*

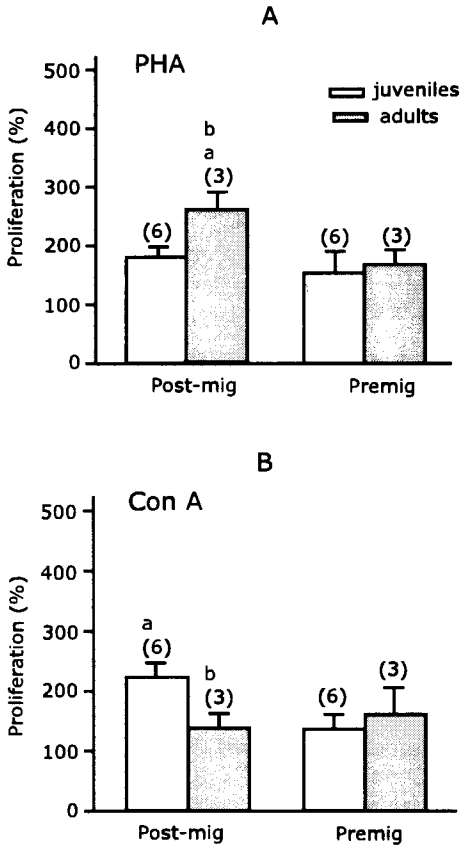

Fig. 2: Effect of the seasonal migratory cycle on the proliferative response of thymic mononuclear leukocytes to the mitogens PHA (A) and Con A (B). The results are the mean ± SEM of the number of animals (in parentheses) on which the determinations were performed in triplicate. a $P<0.05$ versus premigratory; b $P<0.05$ vs. juveniles.

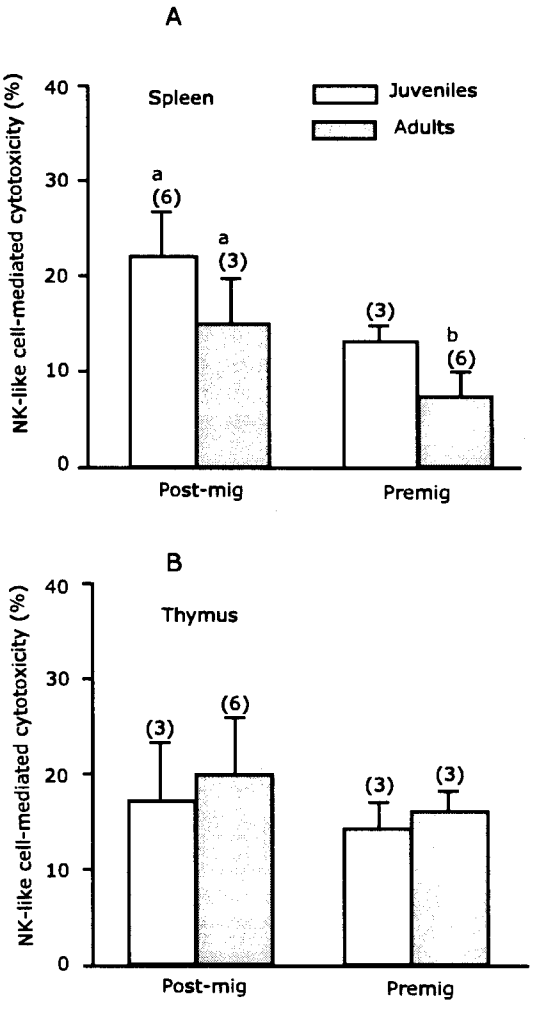

Fig. 3: Effect of the seasonal migratory cycle on NK-like cell-mediated cytotoxicity of splenic (A) and thymic (B) mononuclear leukocytes. The results are the mean ± SEM of the number of animals (in parenthesis) on which the determinations were performed in triplicate. a $P<0.05$ versus premigratory; b $P<0.05$ vs. juveniles.

ridibundus showed NK-like cell-mediated cytotoxicity, as reported in chickens (Haddad and Mashaly, 1992). However, the cytotoxicity results were lower than those reported in mammals (Ferrández and De la Fuente, 1996), likely by a decreased number of NK-like cells in the isolated splenic and thymic mononuclear leukocytes.

Effect of the Migration on Leukocyte Functions: Our results show that the immune functions of splenic and thymic mononuclear cells from *L. ridibundus* are affected by the migratory period. The chemotactic mobility of the mononuclear leukocytes is depleted in the post-migratory period for leukocytes obtained from both spleen and thymus. Similar findings were obtained regarding the response to Con A of splenic mononuclear leukocytes and even with LPS for splenic leukocytes from juveniles. A differential effect of the migratory cycle on the leukocyte subpopulations is suggested since both PHA and Con A are mainly mitogenic for T lymphocytes (Nowell, 1960 and Liener *et al.*, 1996) and LPS and PWM for B lymphocytes (Andersson *et al.*, 1972; Slavatsky and Knulitila, 1989), but the response to these mitogens showed different patterns of response. Thus, we have found

that the proliferation was increased with PHA and PWM for splenic mononuclear leukocytes from juveniles during the post-migratory period. It may be due to a major presence of the PHA- and PWM-responding subpopulations in the spleen after the migratory flight following different patterns of lymphoid recirculation (Butcher, 1986). Regarding the thymus, it was obtained that in the post-migratory period there was a high response to PHA in adults and to Con A in juveniles, suggesting that at least for juveniles the thymus is producing a higher amount of Con A-responding thymocytes as they could be depleted in peripheral tissues after the migratory flight. Moreover, a high cytotoxic activity was observed in the post-migratory period in splenic mononuclear leukocytes. This finding could be a compensatory mechanism to maintain the immune defense of the organism in a stressful situation that is yielding to an immunodepletion of other responses. Furthermore, the effect of the migratory flight could be modulated by hormones and releasing factors that have been reported to modify the lymphoid recirculation and the immune response (Saad, 1988; Davison *et al.*, 1988; Haddad and Mashaly, 1991;Wick *et al.*, 1992). Other factors that could modulate the immune response as low temperatures and photoperiod (Chancellor and Glick, 1960; Spinu and Degen, 1993) were stated as no significant since the conditions of temperature and photoperiod were very similar in both post-migratory and premigratory periods. .

Table 1: Chemotaxis indexes of splenic and thymic mononuclear leukocytes in response to the chemoattractant N-formyl-Met-Leu-Phe (FMLP) during the seasonal migratory cycle of *Larus ridibundus*

	Post-migratory	Premigratory
Juveniles/Spleen	111 ± 13[a] (6)	263 ± 39 (3)
Adults/Spleen	124 ± 10[a] (6)	205 ± 34 (3)
Juveniles/Thymus	143 ± 9[a] (5)	175 ± 17 (3)
Adults/ Thymus	126 ± 8[a] (4)	186 ± 12 (3)

Results are the mean ± SEM of the number of animals (in parentheses) in which the determinations were performed in duplicate. a $P < 0.05$ versus premigratory.

Age-associated Differences: Since an effect of the age on the immune system has been reported in chickens (McCorkle *et al.*, 1990; Lillehoj and Chung, 1992), we were looking for a major depletion in the immune response of juveniles compared to that of adults because of the migratory flight. However we have found that there are significant differences in the response of splenic and thymic mononuclear leukocytes according to the age of the animal, it is not possible to define a pattern of response attending to this parameter. This finding could suggest that compensatory mechanism are also acting in order to avoid a major immunodepletion in the juvenile gulls.

Summarizing, we have found that the immune response of splenic and thymic mononuclear leukocytes from *L. ridibundus* is affected by the migratory annual cycle, although there are different patterns of response. It is probably due to the heterogeneity of the leukocyte subpopulations present in each period.

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