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► **To cite this version:**

M.P. Terrón, J. Delgado, S.D. Paredes, C. Barriga, R.J. Reiter, et al.. Effect of melatonin and tryptophan on humoral immunity in young and old ringdoves. *Experimental Gerontology*, 2009, 44 (10), pp.653. 10.1016/j.exger.2009.07.005 . hal-00520354

HAL Id: hal-00520354

<https://hal.science/hal-00520354v1>

Submitted on 23 Sep 2010

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Accepted Manuscript

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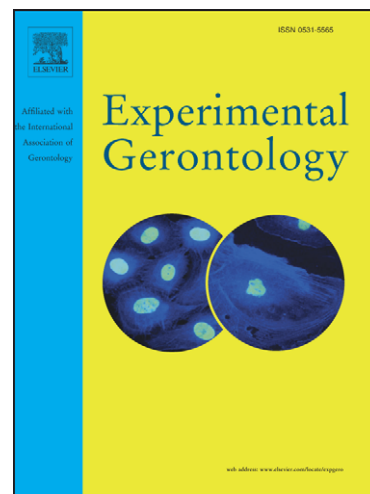
PII: S0531-5565(09)00141-7
DOI: [10.1016/j.exger.2009.07.005](https://doi.org/10.1016/j.exger.2009.07.005)
Reference: EXG 8627

To appear in: *Experimental Gerontology*

Received Date: 5 June 2009
Revised Date: 15 July 2009
Accepted Date: 17 July 2009

Please cite this article as: Terrón, M.P., Delgado, J., Paredes, S.D., Barriga, C., Reiter, R.J., Rodríguez, A.B., Effect of melatonin and tryptophan on humoral immunity in young and old ringdoves (*streptopelia risoria*), *Experimental Gerontology* (2009), doi: [10.1016/j.exger.2009.07.005](https://doi.org/10.1016/j.exger.2009.07.005)

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**EFFECT OF MELATONIN AND TRYPTOPHAN ON HUMORAL IMMUNITY
IN YOUNG AND OLD RINGDOVES (*STREPTOPELIA RISORIA*).**

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Running title: Melatonin on humoral immunity in ringdoves.

Key words: Melatonin, haemolytic and bactericidal activity, immunoglobulins, birds.

ABSTRACT

Melatonin is involved in the regulation of both cellular and humoral immunity. In the present study we have evaluated the effect of the oral administration of melatonin and its precursor, the amino acid tryptophan, on humoral immune response in ringdoves (*Streptopelia risoria*) from different age groups. Male and female ringdoves of 4-5 years of age (young) and 12-14 years of age (old) were used in this study. The animals received a single capsule of 300 mg/kg b.w. of tryptophan (old animals) for 3 consecutive days one hour after lights on or a single oral dose (0.25 or 2.5 mg/kg body weight/0.1 ml per animal/day, young and old animals respectively) of melatonin, for 3 consecutive days 1 hr before lights off. Blood samples were taken before beginning the treatment (basal values) and at the end of the treatment. Immunoglobulins, bactericidal and haemolytic activity were measured. Our results show that in old animals the humoral immune response was reduced with respect to the young. Both melatonin and tryptophan treatment increased the immunoglobulin concentration, with the nocturnal values being significantly higher than diurnal values and with a major effect in old animals. The bactericidal activity of the *Streptopelia risoria* serum against *Staphylococcus aureus*, after the treatment with melatonin or tryptophan, was increased at night with a greater effect in old animals. No significant differences were observed in the haemolytic activity of the serum in young animals, but there was an increase in old animals, with higher values at night after treatment with melatonin. In general, the oral administration of melatonin or tryptophan produced a stimulation of humoral immune response with greater effects in old ringdoves.

INTRODUCTION

The mechanisms employed by the immune system for defending the organism against harmful microorganisms and foreign molecules are coordinated by the environmental 24-h light/dark period in order to achieve optimal efficacy (Skwarlo-Sonta, 2002). Aging is associated with a decline in the immune function (immunosenescence), a situation known to correlate with increased incidence of cancer, infectious and degenerative diseases (Fontana, 2009). Some immunological parameters are commonly significantly reduced in the elderly. Innate, cellular and humoral immunity all exhibit increased deterioration with age (Sansoni et al., 2008). Aging is also associated with a number of changes in the morphology, physiology and biochemistry of a variety of organs including the pineal gland. Alterations in this organ results in a significant reduction of the nocturnal synthesis of its main secretory product melatonin (Reiter et al., 1980, 1981; Pandi-Perumal et al., 2002).

A link between pineal melatonin and immune responses has been established in birds (Moore and Siopes 2000; Moore et al., 2002; Terrón et al., 2005; Singh and Haldar, 2005). Although there are some contrary reports in the literature, most have established an immune enhancing effect of melatonin, especially under conditions when system is compromised. Removal of the primary source of melatonin due to pinealectomy reduces immune responses in the ring dove (Rodríguez and Lea, 1994) and Japanese quail (Moore and Siopes, 2002), suggesting that immune functions are at least partially regulated by physiological levels of melatonin. Moreover, the treatment of these species with exogenous melatonin restores immune function (Rodríguez et al., 2001; Moore and Siopes 2002, 2003; Terrón et al., 2003, 2004; Paredes et al., 2007a; Siopes and Underwood, 2008).

Circulating melatonin decreases with age and in recent years much interest has been focused on the immunomodulatory consequences of this reduction (Olakowska et al., 2005). Melatonin is a natural antioxidant with significant anti-aging properties (Reiter et al., 2008). It is a direct free radical scavenger, an indirect antioxidant via stimulation of antioxidative enzymes and an immunomodulatory agent. Because enhanced oxidative stress plays a crucial role in the aging process and chronic diseases associated with senescence, the administration of a potent amphiphilic antioxidant agent with high bioavailability such as melatonin may become a promising, safe and effective intervention strategy to slow some processes of aging and the initiation and progression of age-related disorders (Poeggerler, 2005).

Melatonin has significant immunomodulatory actions in immune compromised states. Maestroni et al. (1986) was the first to show that inhibition of melatonin synthesis causes a reduction of cellular and humoral responses in mice. Numerous in vivo and in vitro studies have documented that melatonin enhances both natural and acquired immunity in animals (Carrillo-Vico et al., 2005). In our animal model (*Streptopelia risoria*), both the indolamine melatonin and its amino acid precursor, tryptophan, influence activity-rest rhythms, modulate circulating levels of melatonin and serotonin, increase cell viability and resistance to induced oxidative stress in blood heterophils, and likewise enhance the phagocytic function and neutralize superoxide anion radicals generated by heterophils (Paredes et al., 2007b). Also, in the old birds, treatment with melatonin or tryptophan at concentrations and times of administration considered suitable improved nocturnal rest and reversed the immunosuppressive and oxidative effects accompanying phagocytosis at these advanced ages (Terrón et al., 2002; Paredes et al., 2007a,c,d).

Herein, we compared whether exogenous melatonin or tryptophan treatments would have equivalent immune enhancing actions in young and old ring doves.

MATERIALS AND METHODS

Animals

Isolated, male and female ringdoves (*Streptopelia risoria*) of 4-5 years of age (young) or 12-14 years of age (old) were used in this study (n = 10 per age group). The birds were bred in our department, maintained on food and water ad libitum in cages measuring 40x40x45 cm and exposed to 12 h light and 12 h darkness (darkness from 20:00 to 08:00 hours) per day. The temperature was maintained at $22\pm 2^{\circ}\text{C}$ (70% humidity).

The study was approved by the Ethical Committee of the University of Extremadura (Badajoz, Spain) in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Melatonin or tryptophan treatments

Young animals were treated for 3 consecutive days with a single daily oral dose (0.25 mg/0.1 ml phosphate-buffered saline [PBS] per animal) of melatonin (Sigma, St. Louis, MO, USA) at 19:00 h (1 h before the onset of the dark period). Old animals were treated for 3 consecutive days with either a single daily oral dose (2.5 mg/0.1 ml PBS per animal) of melatonin 1 hour before lights off or a single oral capsule of 300 mg/kg of body weight (b.w.) of L-tryptophan (Sigma, St. Louis, MO, USA) at 09:00 h (1 hour after the onset of the light period). The choice of these concentrations and of the time of administration, were based on previous studies, where it was observed that they

restored the amplitude of the serum melatonin rhythm in old doves to that of the young birds, also improving their nocturnal rest, enhanced phagocytosis and free-radical scavenging in both groups of age as well as correcting age-related altered secretion of cytokines and thermoregulatory responses (Paredes et al., 2007 c, d, e, 2009). Control animals received only capsules containing the excipient – methylcellulose (Sigma, St. Louis, MO, USA) – with the same schedule as for the tryptophan-treated animals or 0.1 ml of PBS with the same schedule as in the melatonin-treated animals. Basal values were obtained before treatment from animals that had not been given either methylcellulose, tryptophan or melatonin. As no significant variations were observed in these values with respect to the controls, they are not reported herein.

Serum collection

Blood samples were drawn from all ringdoves at the melatonin acrophase (times at which the variable reached their maxima) and nadirs (times at which the variable reach their minima) allowing at least one week between consecutive extractions. The acrophases of the melatonin rhythm in the basal groups were established at 02:00 h and 01:00 h, and nadirs at 14:00 h and 13:00 h, in young and old ringdoves, respectively (Paredes et al., 2006). In the melatonin treatment birds, the acrophase and nadirs were respectively established at 02:30 h and 14:30 h and 03:00 h and 15:00 h in young and old animals (Paredes et al., 2007d). In the tryptophan-treated animals, the acrophase and nadir were established at 03:30 h and 15:30 h, respectively (Paredes et al., 2007e). Blood was extracted (1 ml per animal per week) using a 25-gauge needle and a 1 ml syringe from the brachial vein and transferred unheparinized to a pre-prepared tube containing serum-separating gel. The samples were centrifuged at room temperature for 30 min at 300 x g. The serum was then divided into aliquots in Eppendorf vials, and

kept frozen at -30°C until the time of assay. Nocturnal collections were performed under dim red light which is perceived as darkness. The extractions were performed before beginning the treatment (basal values) and after the 3 days of treatment.

Bacteria

The bacteria used were *Staphylococcus aureus* (ATCC 9144) obtained from the Department of Microbiology, Faculty of Medicine, Badajoz.

Serum immunoglobulin determinations

Serum immunoglobulin (Ig) levels (isotypes A, M, and G) were measured following administration of a 0.1 ml intravenous injection of sheep erythrocytes suspension (Akbulut et al., 2001) into the brachial vein at the end of the study period and before the last melatonin injection or tryptophan capsule, in order to evoke humoral immune responses; the birds in the control groups were given 0.1 ml of phosphate buffered saline (PBS). Total serum immunoglobulin were measured with a chicken serum IgA, IgG or IgM enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX, USA) following the manufacturer's protocol.

Serum bactericidal activity

The technique used was previously described by Barriga et al. (1994). The serum was diluted in Mueller Hinton Broth (MHB) to a titre of 1:16 with a final tube volume of 100 μl . Then, 10 μl of 1×10^5 CFU (Colony-forming units) of *Staphylococcus aureus* per ml were added. In addition, there was a tube with MHB and bacteria inoculum but without serum. The content of the tubes was seeded onto Mueller-Hinton agar plates and incubated at 37°C , 100% humidity, and 5% CO_2 . After 24 h, CFUs were

determined and results expressed as a growth percentage (CFU%) by means of the expression:

$$\text{CFU (\%)} = \frac{\text{CFU samples with serum}}{\text{CFU samples without serum}} \times 100$$

Where 100% (maximum growth) represents the number of CFU surviving in a basal samples where the bacteria had been incubated in the absence of serum.

Serum haemolytic activity

Serum haemolytic activity was evaluated using a colorimetric assay for the quantitative determination of complement function in serum (NovaTec, Immundiagnostica GMBH, Dietzenbach, Germany) which consisted of a reference serum, incubation buffer, immunocomplex, ONPG (o-nitrophenilgalactopiranoside) substrate solution, etandiol and stop solution, following the manufacturer's protocol. Results are expressed as CH-50 units. The NovaTec CH 50 method is based on the capacity of complement to solubilise the immunocomplex.

Statistical analysis

Data are expressed as mean \pm S.E.M. (Standar Error of Mean) of the number of determinations carried out in duplicate. The results were analysed using a non-parametric one-way ANOVA, followed by a *post hoc* Tukey test to compare all pairs of columns, at the significance level $2\alpha= 0.05$.

RESULTS

As no significant differences were observed between the results obtained in the basal measures (serum levels before treatment) and levels from vehicle-treated birds or control birds (animals treated with PBS or a capsules containing the excipient – methylcellulose), the basal values are omitted from the results.

Figure 1A shows the serum levels of immunoglobulin A (IgA) in control (PBS or methylcellulose -treated) and after 3 days of melatonin or tryptophan treatment for both young and old animals. In young control animals, the nocturnal levels were not statistically different than the diurnal values. After treatment with melatonin, neither the nocturnal nor the diurnal levels of IgA were changed. Also, in old animals there were no significant differences in the diurnal or nocturnal control levels and the young control values. The diurnal values obtained after the treatment with tryptophan remained unchanged with respect to their control. However there was a significant decrease ($p<0.05$) in the diurnal level after melatonin treatment and in the nocturnal levels with both tryptophan and melatonin treatment.

Figure 1B shows the serum levels of immunoglobulin G (IgG) in control (PBS or methylcellulose -treated) and after 3 days of melatonin or tryptophan treatment for both young and old animals. There was no difference between the non-treated and melatonin-treated young animals. However, in old birds, the administration of melatonin or tryptophan caused a significant increase in diurnal and nocturnal values ($p<0.05$) with respect to their control values at night.

Treatment with melatonin (in both young and old animals) or tryptophan (old animals) caused a significant elevation ($p<0.05$) in the serum levels of immunoglobulin M (IgM) (Figure 1C) in both the day and night, when compared them to their controls. Values obtained in old animals were lower than those in the young birds.

The bactericidal activity of the *Streptopelia risoria* serum, from young and old animals non-treated and treated with melatonin or tryptophan, against *Staphylococcus aureus* are represented in Figure 2. There was a significant elevation ($p < 0.05$) in the bactericidal activity with respect to the basal value (the bacteria incubated in the absence of serum) in both age groups studied and either non-treated (control values) or treated animals (melatonin or tryptophan values). The values obtained for the melatonin treated young birds were higher than their control values. Serum from old birds receiving tryptophan or melatonin treatment exhibited an increase in bactericidal activity with respect to their control values (serum obtained from birds PBS or methylcellulose -treated); higher values were seen at night after the treatment with melatonin.

The effect of exogenous melatonin or tryptophan on the haemolytic activity of serum from different age groups (*Streptopelia risoria*) is shown in Figure 3. Exogenous melatonin did not change the haemolytic activity of serum from young animals compared to the values observed in controls birds. In old birds, there was an increase in the number of CH-50 units after both tryptophan or melatonin treatment. The values were significantly higher ($p < 0.05$) at night.

DISCUSSION

Aging is a complex process that involves multiple re-organisational and developmentally regulated changes. Many immunological parameters are reduced significantly in the elderly. These age-associated immune dysfunctions are the consequence of declines in the generation of lymphocytes, the reduction in the activity of interleukin-2, an increase in apoptosis, the decrease in the CD4/CD8 cell ratio and a change in the proportion of virgin to memory cells (Sansoni et al., 2008). Especially, a significant loss of the number of antigen-specific CD+ T-cells in aging has been reported (Kovaiou and Grubeck-Loebenstein, 2006). It is suggested that humoral responses are reduced due to poor T helper function and increased anti-idiotypic down-regulation in aged animals (Hakim and Gress, 2007).

In the present study, in order to determine the immunoglobulin serum concentration, birds were immunized against sheep erythrocytes (SRBC). In the control groups, the immunoglobulin (Ig) levels of aged birds were lower than serum levels of young birds. This reduction was even more pronounced when we measured the IgM levels. This finding suggests that the humoral response of young animals is higher than that of aged ones.

Melatonin is a well established agent which is known to exert positive effects on the cellular and humoral immune response in mammals and birds. Melatonin is produced and secreted into the blood in a circadian manner with maximal plasma levels occurring during the dark phase of the light/dark cycle (Reiter, 1991). The role of the pineal gland, suprachiasmatic nucleus (SCN), and clock genes for the induction and regulation of circadian rhythms is important and are well-documented (Berger, 2008). Whereas the 24-hour rhythm of melatonin production is clear in young birds, this cycle deteriorates with age (Skwarlo-Sonta, 1996). As in other laboratories, we have reported

that the daily oscillations of the levels of melatonin in young birds, also is reduced in old ring doves (Terrón et al., 2002; Paredes et al., 2006).

In the current study, exogenous melatonin or tryptophan significantly increased the IgM levels in the aged birds. In reference to the other subtypes of immunoglobulins evaluated, there was a significant reduction in IgA at night after the treatment with tryptophan and both during day and at night when melatonin was given. No differences were observed in IgG serum levels after the treatments. The results reported here are consistent with most of the existing literature, which indicates an immunoenhancing effect of melatonin, specially on IgM levels, in both mammals and birds (Carrillo-Vico et al., 2005).

Akbulut et al., (2001) investigated the age-dependent changes in the humoral immune response and the effects of short-term use of the pharmacological doses of melatonin on this response in rats and concluded that exogenous melatonin may augments depressed humoral immune response seen in aged animals. In addition, Moore and Siopes (2002, 2003) studied the immunoenhancing effect of melatonin on cellular and humoral response in birds without prior immunosuppression and via an opiategic mechanism, concluding that melatonin enhances cellular and humoral response in these birds. Subsequently, Siopes and Underwood (2008) studied the role of melatonin in the diurnal variation in the cellular and humoral immune response of Japanese quail and concluded that there exists a melatonin dependent diurnal variation in both cellular and humoral immune response in quail.

Complement is involved in the most important system of lysis in humoral immunity. This system is activated via two different pathways: the classical and the alternative. The purpose of the present study has been to evaluate whether exogenous melatonin or tryptophan act on this important defence system via either of these

pathways. To test the function of the classical pathway, we used the haemolytic activity test. The results show that the administration of melatonin in young animals did not influence the CH-50 units with respect to their control values. However there was a significant increase of the haemolytic activity when old animals were given melatonin or tryptophan; the highest values were measured at night and with melatonin administration. These results indicate that the serum haemolytic activity is stimulated by both exogenous melatonin or tryptophan.

The alternative complement pathway is a major defensive system against Gram-negative bactericidal invasion in vertebrates. This pathway was evaluated by means of the bactericidal mechanism present in the serum, expressing the results as the percentage of CFU that survive when the bacteria are incubated for 24 hours in the presence of serum, with the basal value (bacteria incubated without serum) being 100%. A significant rise was found in the values of bactericidal activity when the animals were treated with exogenous melatonin or tryptophan with respect to the basal values and even with respect to their control values (bacteria incubated with serum before treatment). We believe this is the first account of a melatonin effect on haemolytic and bactericidal activity.

Our results show that in old animals the humoral immune response declined. The administration of melatonin or its precursor tryptophan to birds immunized against SRBC enhanced the humoral immune response as shown by the increase of blood immunoglobulins levels especially in old animals at night; this would improve their efficiency during aging when immune function normally declines.

Many other reports have indicated that melatonin and tryptophan exert a modulatory effect on immune system, especially in old animals. In this sense, our demonstrated results have demonstrated that melatonin has immuno-stimulatory

properties (Terrón et al., 2002, 2003, 2004, 2005) and is able to modulate certain immune functions (Rodríguez et al., 2001; Maestroni et al., 1988; Skwarlo-Sonta, 2002). These data show that melatonin enhances both natural and acquired immunity.

Currently, it is known that treatment with this exogenously-administered indoleamine restores the reduced immunological functions. Our most recent studies documented that exogenous melatonin enhances heterophil viability in old animals as well as increasing phagocytosis and free-radical scavenging in both age groups during the nocturnal period, accompanied by an increase in the levels of the indoleamine (Paredes et al., 2007a,c), and not only melatonin but tryptophan, may also have immuno-enhancing effects. Studies have also tested the effect of tryptophan on the phagocytic response. Thus, we observed that tryptophan treatment significantly increased serum melatonin and serotonin levels, cell viability, phagocytosis index and phagocytosis percentage. Moreover, we found that tryptophan intake increased melatonin levels in plasma and pineal over a 24-hour period (Sanchez et al., 2008). Superoxide anion levels decreased significantly with respect to vehicle values, with the nocturnal reduction being greater than that which occurred during the light period (Paredes et al., 2007d).

Previously, we found that melatonin administered to old ringdoves increased the differences between the nocturnal and diurnal plasma levels of the indoleamine, and in parallel, enhanced phagocytosis and reduced the levels of the superoxide radical in the heterophils (Terrón et al., 2005). These findings confirmed earlier reports of a positive melatonin-phagocytic activity in vitro of heterophils from mature and old animals after incubation of the cells with either physiological or pharmacological concentrations of melatonin (Terrón et al., 2002, 2003).

In this study, it was found that melatonin and its precursor tryptophan, restored the suppressed immunoglobulins levels in old ringdoves; this suggests that both of these constituents play an important role in maintaining normal immune function. These observations suggest that melatonin and tryptophan treatment may be successfully used in the therapy of immunodepressive conditions.

ACCEPTED MANUSCRIPT

Acknowledgements

The authors would like to express their thanks to Ms. Elena Circujano Vadillo for her technical assistance and to Manuel Molina and Manuel Mota of the Department of Mathematics (Section of Statistics and Operations Research) of the Faculty of Science for their advice and collaboration in the statistical analyses of the data. This investigation was supported by a research grant from Consejería de Infraestructuras y desarrollo Tecnológico (Junta de Extremadura, 3PRO5A053). M.P. Terrón and S.D. Paredes were beneficiaries of grants from the Consejería de Economía, Comercio e Innovación – Fondo Social Europeo (Junta de Extremadura, REI07004 and POS07012 respectively).

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FIGURE LEGENDS

Figure 1. Serum levels of IgA (A), IgM (B) and IgG (C) in control (methylcellulose or PBS-treated) young and old animals and in birds treated with melatonin or tryptophan.

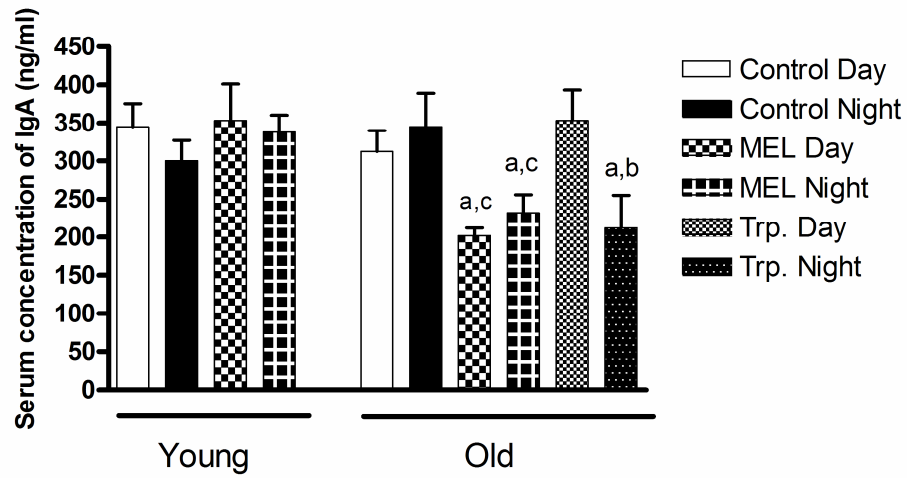
Each value represents the mean \pm S.E.M. of ten determinations performed in duplicate.

a) $p < 0.05$ with respect to their control. b) $p < 0.05$ with respect to their daily values. c) $p < 0.05$ with respect to the values obtained in young animals. Trp.= Tryptophan. MEL= melatonin.

Figure 2. Bactericidal activity of serum from *Streptopelia risoria* of different ages, against *Staphylococcus aureus*. Each value represents the mean \pm S.E.M of ten determinations performed in duplicate. *) $p < 0.05$ with respect to the basal (without serum) values. Trp.= Tryptophan. MEL= melatonin. CFU= Colony-forming units.

Figure 3. Haemolytic activity (CH-50 units) of serum from young and old *Streptopelia risoria*. Each value represents the mean \pm S.E.M. of ten determinations performed in duplicate. a) $p < 0.05$ with respect to their control. b) $p < 0.05$ with respect to their daily values. c) $p < 0.05$ with respect to the values obtained in young animals. Trp.= Tryptophan. MEL= melatonin.

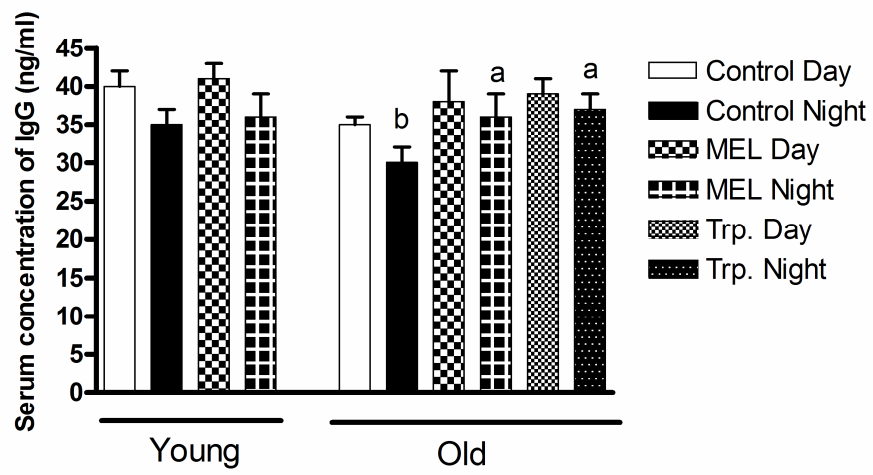
Figure 1A



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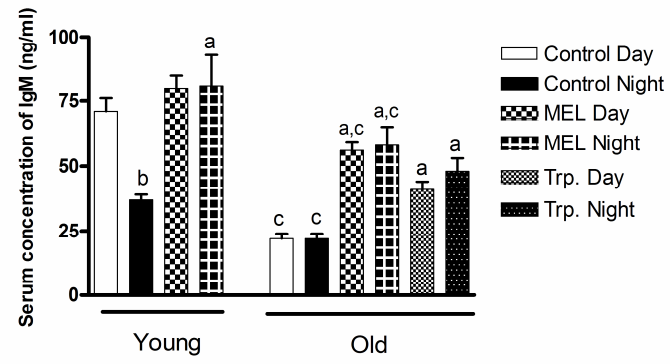
Figure 1B



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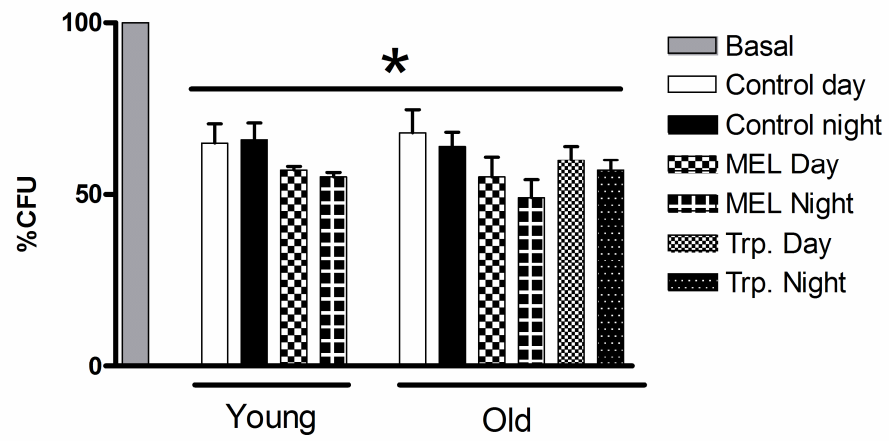
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Figure 1C



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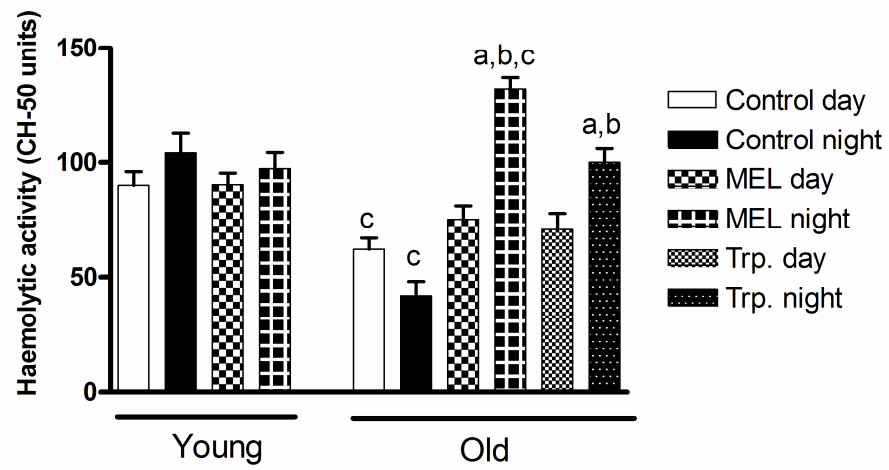
Figure 2



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Figure 3



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