

# Circadian effects on the acute responses of salivary cortisol and IgA in well trained swimmers

L Dimitriou, N C C Sharp, M Doherty

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**Objective:** To examine whether time of day significantly affects salivary cortisol and IgA levels before and after submaximal swimming.

**Methods:** Fourteen male competitive swimmers (mean (SD) age 18 (3.2) years) volunteered to participate in the study. In a fully randomised, cross over design, each subject performed 5 × 400 m front crawl at 85 (1.2)% of their seasonal best time (277 (16) seconds), with one minute rest between each 400 m, at 0600 and 1800 hours on two separate days. Timed, unstimulated saliva samples were collected before and after exercise. Saliva samples were analysed for cortisol and IgA by radioimmunoassay and single radial immunodiffusion respectively.

**Results:** Significant time of day effects (am and pm respectively) were observed in IgA concentration (0.396 (0.179) v 0.322 (0.105) mg/ml,  $p < 0.05$ ), IgA secretory rate (0.109 (0.081) v 0.144 (0.083) mg/min,  $p < 0.01$ ), and saliva flow rate (0.31 (0.23) v 0.46 (0.22) ml/min,  $p < 0.001$ ) before exercise (all values mean (SD)). Differences in cortisol levels before exercise (1.09 (0.56) v 0.67 (0.94) µg/dl) approached significance ( $p = 0.059$ ). The exercise protocol did not significantly affect IgA concentration and secretory rate ( $p > 0.05$ ) but, in comparison with values before exercise, caused significant alterations in cortisol ( $p < 0.01$ ) and saliva flow rate ( $p < 0.01$ ). There was no significant interaction effect of time of day by exercise on any salivary variables measured ( $p > 0.05$ ). However, most of the values of the salivary variables before exercise were significantly inversely related to their exercise induced response ( $p < 0.05$ ).

**Conclusion:** These results suggest a significant circadian variation in the variables measured before exercise, without showing a significant effect on their acute responses to exercise.

See end of article for authors' affiliations

Correspondence to:  
L Dimitriou, Department of Sport Sciences, Brunel University, Osterley Campus, Borough Road Isleworth, Middlesex TW7 5DU, UK;  
Lygeri@hotmail.com

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Virtually all physiological and psychological variables show evidence of large rhythmic changes (24 hour rhythmicity),<sup>1,7</sup> and many physiological responses to exercise are influenced by the time of day effect.<sup>1,2</sup> However, the time of day effects on the immunoendocrinological responses to exercise are not clear.<sup>8</sup> Studies on salivary cortisol (an immunosuppressive hormone) and IgA (the first line of defence against upper respiratory infections) in response to exercise appear equivocal,<sup>9–20</sup> yet these variables have been used extensively for monitoring athletes' health. A possible explanation for the variability found in the literature could be the difference in time of day at which the measurements were taken. The persistence of circadian rhythms during exercise conditions could result in erratic interpretation of the body's responses in sporting and clinical research and could invalidate experiments in which measurements were not taken at a consistent time of day.<sup>3,9</sup> It could also have implications for exercise regimens that could lead to an overload of the immune system. Furthermore, the effects of circadian variation and exercise on potential triggers of immunosuppression may be synergistic. For example, exercising in the morning could possibly increase susceptibility to infection compared with an afternoon training session. It is documented that cortisol secretion peaks in the early morning,<sup>2,5</sup> and the rate of salivary IgA secretion is conversely at its nadir during this time.<sup>6</sup> A trawl of articles on the effects of circadian rhythms on the acute response of the mucosal immune system and salivary cortisol after swimming shows a dearth of information. Providing more information on this subject could help to identify the optimal time for training in terms of the least immunosuppressive effects and to emphasise the significance of obtaining experimental and control data at identical time points.

This study was conducted to determine the time of day effects, (morning (0600) v evening (1800)) before and after intermittent submaximal swimming, on salivary cortisol and IgA in competitive male swimmers. It was hypothesised that (a) the variables examined would show a circadian variation between morning and evening, and (b) their responses to exercise would be determined by their basal levels measured before exercise.

## METHODS

### Subjects

Fourteen healthy male competitive swimmers, who all habitually trained for 90–120 minutes in both the morning and evening, volunteered. They had a mean (SD) age of 18.81 (3.19) years, height of 1.79 (5.98) m, and weight of 70.78 (6.60) kg. They had been training for a mean (SD) of 8.68 (2.02) years, with a swimming distance of 21.34 (3.71) km/week and a seasonal best time (PBT) of 277 (16) seconds. Two were club standard, one was county standard, two were regional standard, and nine were national standard. Assessment of their circadian chronotype characteristics showed that 11 were intermediate (neither morning nor evening type), two were moderate evening types, and one was a definite evening type.

A full verbal and written description of all procedures and requirements of the study was given to all subjects before they or their parents signed a Brunel University ethics committee approved consent form. In addition, an information pack to remind the subjects of the behavioural restrictions, food intake, and activity record tables, as well as the questionnaires on personal details and chronotype,<sup>7</sup> were given to the subjects before testing.

### Experimental design

In a fully randomised, cross over design, each subject performed two tests at 0600 and 1800 hours in the same indoor heated pool (water temperature 28.7 (0.5)°C, air temperature 26.6 (1.2)°C, and humidity levels 70.3 (5.8)%), with a minimum of 36 hours between the test sessions. The maximum time between the tests for any one subject was eight days. The time of day for testing was selected to correspond closely to the circadian peaks and troughs of most of the variables measured,<sup>2 5 6</sup> and, coincidentally, are the most common times for swimming training.

This study was designed to eliminate many of the physiological variables known to cause alterations in salivary cortisol and IgA.<sup>15 16 21–24</sup> Subjects were instructed to refrain from any intense physical activity, dietary supplements, and medicines for 24 hours before the test session.<sup>15 21</sup> Furthermore, sex, tobacco, alcohol, and caffeine consumption were restricted for 12 hours, and eating for eight hours, before testing.<sup>22</sup> Drinking water, chewing gum or mints, and teeth brushing were prohibited one hour before testing. Subjects were also instructed not to disrupt sleep patterns. On the night preceding each exercise test, each subject slept for a mean (SD) of 7.2 (1.5) hours. The last meal (eight hours before testing) was either plain pasta or rice only.<sup>21</sup>

On the first test day, the weight (Tefal scales; 0–120 kg, ± 500 g) and height (Harpenden stadiometer) of the subjects were measured. Mood was evaluated once before the swimming test and again about 25 minutes after, using the POMS-C questionnaire.<sup>25</sup> A saliva sample was also collected at this time.

### Warm up

As a warm up, a 600 m swim was completed at each subject's preferred pace together with their chosen flexibility exercises. In addition, 2 × 100 m of front crawl was performed at the average split time of the 400 m target time to familiarise the subjects with target time and pace. The warm up was kept constant within subjects over the two testing sessions.

### Swimming test

The swimming test consisted of 5 × 400 m front crawl repeats swum at 85 (1.2)% of their season's best time (277 (16) seconds) with a one minute rest between each 400 m. Time was recorded (0.01 seconds) with a digital hand stopwatch. At the end of each 400 m, subjects were instructed to indicate their rating of perceived exertion (RPE, 0–10 Borg scale<sup>26</sup>). At the end of the 5 × 400 m, swimmers exited the pool, dried themselves, and prepared for salivary sampling. The POMS-C and RPE tests were used to control psychological factors known to influence cortisol and IgA before, during, and after exercise.<sup>16 23 24</sup>

### Saliva sampling

Before saliva collection, subjects were required to rinse out their mouths for one minute with water to remove any substances such as chlorine that may affect cortisol and/or IgA levels.<sup>21</sup> Timed unstimulated saliva samples were collected in prechilled and preweighed plastic universal containers (20 ml) before the warm up of the swimming test and seven minutes after termination of the swimming test on both test occasions. Immediately after sampling, samples were frozen and stored at –20°C until assayed for IgA and cortisol concentrations.<sup>9</sup>

Although blood has been traditionally used for cortisol assessment,<sup>27</sup> saliva reflects the level of unbound cortisol more accurately than serum total cortisol. Unbound cortisol, as in saliva, gives a direct measurement of the biologically available molecule,<sup>22</sup> whereas bound cortisol, as in serum, is physiologically inactive.<sup>28</sup> In addition, measurement of serum cortisol requires venepuncture, which is associated with negative feelings, which could increase cortisol<sup>27</sup> and decrease IgA.<sup>29 30</sup>

### Cortisol assay

After being thawed, saliva samples were analysed for cortisol using the radioimmunoassay "Magic cortisol Component Set" (Chiron Diagnostics Corporation, Norwood, Massachusetts, USA). A 90 µl sample of the standard was pipetted into tubes, followed by 100 µl antibody and 50 µl tracer. After incubation at room temperature for 3.5 hours, magnets were attached to the tubes for five minutes. The tubes were then decanted into a pan, and the radioactivity bound to them was counted in a gamma counter for two minutes.

### Saliva flow rate (Saliva<sub>fr</sub>)

The Saliva<sub>fr</sub> was calculated by dividing the sample volume (ml) by the time (min) taken to produce it.<sup>13</sup> For the estimation of Saliva<sub>fr</sub>, it was assumed that saliva density was 1.00g/ml.<sup>20</sup>

### IgA secretion rate (IgA<sub>sr</sub>)

IgA<sub>sr</sub> was calculated by multiplying the absolute IgA concentration (mg/ml) by the Saliva<sub>fr</sub> (ml/min).<sup>13</sup>

### IgA assay

Secretory IgA concentrations were determined by single radial immunodiffusion<sup>31</sup> using a Bind A Rid kit (Binding Site Limited, Birmingham, UK). Calibrators and control and saliva samples (thawed) were mixed immediately before use, and 10 µl of each were pipetted into wells containing monospecific antibody in an agarose gel, and incubated at room temperature for four days. High IgA concentrations were diluted 1:2 with 1% sheep albumin. Precipitin ring diameters were measured with an eyepiece graticule. A calibration curve from the samples containing known amounts of antigen (mg/l) was constructed, and the antigen concentrations were determined by squaring the ring diameters and interpolating them from the standard curve. Note that a radial immunodiffusion reference table for IgA was also used.

### Statistical analysis

All values reported are mean (SD). Data showing skewness and kurtosis were transformed to natural logarithms (ln). This logarithmic transformation of skewed and/or kurtotic data was undertaken to improve the statistical inference of the tests and further attenuate the impact of outliers.<sup>32</sup> A two factor (time of day (0600 hours, 1800 hours)) × (exercise conditions (before and after exercise)) repeated measures of multivariate analysis of variance and analysis of variance was applied to the data of POMS-C, salivary cortisol, IgA, IgA<sub>sr</sub>, and Saliva<sub>fr</sub>. This was to examine: (a) whether time of day significantly affects values before exercise; (b) the effects of exercise on the selected variables; (c) the interaction of time of day with their exercise induced responses. One way multivariate analysis of variance was used to determine whether time of day significantly affected RPE.

Pearson product moment correlations were pursued between baseline values and exercise induced response of cortisol, IgA<sub>sr</sub>, and Saliva<sub>fr</sub> in the morning and evening.

The change in values for the variables caused by exercise was calculated by subtracting the value obtained before exercise from that obtained after exercise. *p* was set at <0.05.

## RESULTS

### Salivary cortisol

Cortisol levels measured before exercise did not show a significant time of day variation. However, after exercise there was a significant increase in cortisol levels. No significant interaction effect between time of day and exercise on cortisol was observed (table 1).

Table 1 Cortisol ( $\mu\text{g/dl}$ ),  $\text{IgA}_{\text{sr}}$  (mg/min), IgA concentration (mg/ml) and  $\text{Saliva}_{\text{fr}}$  (ml/min) measured before and after exercise at 0600 (am) and 1800 (pm)

Variable		Before exercise	After exercise	Absolute change (SD)	Change (%)	Time effect	Exercise effect	Time $\times$ exercise interaction
Cortisol	am	1.09 (0.56)	1.32 (0.67)	$\uparrow$ 0.23 (0.99)	21.1	$F=4.277$	$F=5.963$	$F=0.288$
	pm	0.67 (0.94)	1.18 (0.73)	$\uparrow$ 0.51 (1.26)	76.1	$p=0.059$	$p=0.030^*$	$p=0.600$
$\text{IgA}_{\text{sr}}$	am	0.109 (0.08)	0.076 (0.04)	$\downarrow$ 0.032 (0.09)	9.4	$F=14.665$	$F=4.158$	$F=0.025$
	pm	0.144 (0.08)	0.105 (0.06)	$\downarrow$ 0.038 (0.10)	6.3	$p=0.002^{**}$	$p=0.062$	$p=0.877$
IgA	am	0.396 (0.18)	0.443 (0.20)	$\uparrow$ 0.047 (0.26)	11.9	$F=4.77$	$F=0.70$	$F=0.104$
	pm	0.322 (0.10)	0.344 (0.14)	$\uparrow$ 0.023 (0.15)	7.1	$p=0.048^*$	$p=0.418$	$p=0.752$
$\text{Saliva}_{\text{fr}}$	am	0.31 (0.23)	0.19 (0.10)	$\downarrow$ 0.12 (0.23)	38.7	$F=22.93$	$F=13.844$	$F=0.041$
	pm	0.46 (0.22)	0.33 (0.16)	$\downarrow$ 0.14 (0.16)	30.4	$p=0.000^{***}$	$p=0.003^{**}$	$p=0.843$

Values are mean (SD) ( $n=14$ ).

\*Significantly different at  $p<0.05$ , \*\*significantly different at  $p<0.01$ , \*\*\*significantly different at  $p<0.001$ .

$\text{Saliva}_{\text{fr}}$ , Flow rate of saliva;  $\text{IgA}_{\text{sr}}$ , rate of IgA secretion;  $\uparrow$ , increase;  $\downarrow$ , decrease; Change, post-exercise value – pre-exercise value.

### $\text{IgA}_{\text{sr}}$

$\text{IgA}_{\text{sr}}$  before exercise showed a significant time of day variation. Exercise did not significantly affect  $\text{IgA}_{\text{sr}}$ . No significant interaction effect was observed between time of day and exercise induced effects on  $\text{IgA}_{\text{sr}}$  (table 1).

### IgA concentration

Before exercise, IgA concentration also showed a significant time of day variation. Exercise did not have a significant effect on IgA concentration. No significant interaction was observed between time of day and exercise induced effects on IgA concentration (table 1).

### Saliva<sub>fr</sub>

A significant chronobiological oscillation of  $\text{Saliva}_{\text{fr}}$  before exercise was found. Submaximal swimming significantly reduced the quantity of saliva produced at both times with a mean decrease of 0.13 ml/min. No significant interaction was observed between time of day and exercise induced effects on  $\text{Saliva}_{\text{fr}}$  (table 1).

### Swimming times and intensity

One way multivariate analysis of variance with repeated measures indicated that no significant differences were observed between the two  $5 \times 400$  m swims (326.4 (19.9) seconds in the morning v 326.5 (20.2) seconds in the evening;  $T^2 = 0.111$ ,  $F_{1,13} = 0.209$ ,  $p = 0.95$ ). Thus the two sessions were of similar intensity (84.8 (1.2)% of PBT in the morning v 84.8 (1.3)% of PBT in the evening;  $T^2 = 0.091$ ,  $F_{1,13} = 0.164$ ,  $p = 0.97$ ).

### RPE

One way multivariate analysis of variance with repeated measures of RPE (0–10 Borg scale) indicated a non-significant effect of time of day (am v pm) on the perception of exertion (5.7 (2.0) v 5.5 (1.7);  $T^2 = 0.946$ ,  $F_{1,13} = 1.703$ ,  $p = 0.230$ ).

### Profile of mood states

A two way multivariate analysis of variance with repeated measures on the POMS-C data showed that there was no significant circadian rhythm in perception of mood states between morning and evening under the instructions of "How do you feel right now?" ( $T^2 = 0.434$ ,  $F_{6,13} = 0.578$ ,  $p = 0.740$ ) before and after exercise. Collectively, global mood measured before exercise was better in the morning than in the evening. Exercise did not significantly affect global mood ( $T^2 = 1.326$ ,  $F_{6,13} = 1.768$ ,  $p = 0.223$ ). No significant interaction effect was found for exercise by time on the data of global mood ( $T^2 = 1.604$ ,  $F_{6,13} = 2.139$ ,  $p = 0.223$ ).

## DISCUSSION

The results confirm the existence of circadian variation in the salivary variables, which supports the first hypothesis. However, swimming exercise intensity and subjects' mood state (before and after exercise) and perceived exertion during exercise did not show significant variations between the morning and evening tests ( $p>0.05$ , table 1). This is important to note because many studies have shown that factors such as exercise intensity,<sup>16 22 33</sup> mood state,<sup>16 24 33</sup> and perception,<sup>24</sup> do have a significant effect on cortisol levels, IgA concentration and  $\text{IgA}_{\text{sr}}$ . As most of the influential factors were either controlled or met, the data reported probably indicate interaction of circadian rhythms and exercise. When the same statistical analysis was performed on both raw and ln data (cortisol,  $\text{IgA}_{\text{sr}}$ , and POMS-C), similar results were found. However, analysis of cortisol levels before exercise between morning and evening showed significant differences between raw data ( $p = 0.059$ ) and ln data ( $p = 0.010$ ).

### Cortisol

No significant circadian variation was observed in salivary cortisol levels before exercise ( $p>0.05$ , fig 1). However, in line with previous work,<sup>2 5 7 34</sup> cortisol was higher in the morning than evening, possibly to promote gluconeogenesis and appetite.<sup>34</sup> Exercise significantly increased cortisol levels (fig 1), possibly explained as the end product of hypothalamic-pituitary-adrenal axis activity stimulated in turn by the intensity and duration of the swimming test. Previous studies have shown that exercise at more than 60% of maximum oxygen intake increases cortisol levels.<sup>12 15 19</sup> However, of the 28 post-exercise measures, a decrease in cortisol after exercise was observed, morning and evening as has been shown elsewhere.<sup>16</sup> This decrease in cortisol after exercise can be explained by negative feedback regulation.<sup>9 33</sup> However, the large intersubject variability in cortisol levels makes interpretation difficult.<sup>11 35</sup> This suggests it is unsuitable as a monitoring variable for groups of athletes, although it may prove useful for monitoring individuals.

Despite the fact that the exercise induced cortisol response was found to be greater (76.1%) in the evening than the morning (21.1%), circadian rhythms did not have a significant effect on the magnitude of the cortisol response to submaximal swimming, which is in line with other studies,<sup>15 36</sup> (table 1). This indicates that exercise induced cortisol response is independent of variations throughout the day. However, the significant inverse relations observed between the pre-exercise cortisol levels and the exercise induced cortisol response (cortisol level measured after exercise minus level before exercise, am and pm;  $r = -0.763$ ,  $p = 0.002$  and  $r = -0.817$ ,  $p = 0.000$  respectively) suggest that the exercise induced response of

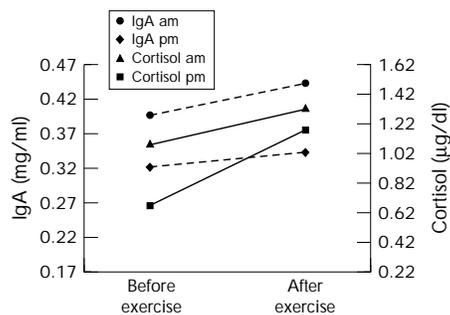


Figure 1 Mean salivary cortisol and IgA levels measured before and after exercise at 0600 (am) and 1800 (pm). There was a significant difference in salivary IgA concentration before and after exercise ( $p < 0.05$ ). There was a significant difference in salivary cortisol concentration measured before exercise between morning and evening ( $p < 0.05$ ).

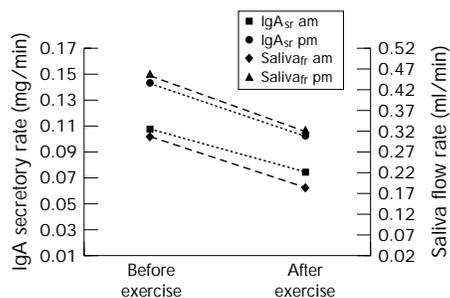


Figure 2 Mean salivary IgA secretory rates and saliva flow rates measured before and after exercise at 0600 (am) and 1800 (pm). There was a significant difference in salivary IgA secretory rate measured before exercise in the morning and evening ( $p < 0.01$ ). There was a significant difference in saliva flow rate measured before exercise in the morning and evening ( $p < 0.001$ ). There was a significant difference in saliva flow rate measured before and after exercise ( $p < 0.01$ ).

salivary cortisol is primarily dependent on the baseline levels measured before exercise, which in turn are mainly regulated by circadian rhythms. The fact that time of day was not found to significantly affect the exercise induced cortisol response may be attributable to the small number of subjects used together with the considerable intersubject variability noted before and after exercise.

#### IgA concentration, $IgA_{sr}$ and $Saliva_{sr}$

In this study, IgA concentration,  $IgA_{sr}$  and  $Saliva_{sr}$  before exercise showed significant time of day variations, which supports the theory that the mucosal immune system is affected considerably by circadian rhythms.<sup>2, 37</sup> IgA concentration before exercise was found to be high in the morning and low in the evening (figs 1 and 2), which is consistent with findings from previous work.<sup>6</sup>

$IgA_{sr}$  (mg/min) and  $Saliva_{sr}$  measured before exercise were found to be higher in the evening than in the morning in contrast with IgA concentration (figs 1 and 2). This may be explained by sympathetic nerve activity, which is higher in the morning than at other times of the day.<sup>7, 38</sup> Increased sympathetic activity decreases  $Saliva_{sr}$ , whereas parasympathetic activity increases it.<sup>29, 39</sup> Furthermore, many steroid hormones such as cortisol have been found to affect the composition—for example, IgA, electrolytes—and secretion rate of saliva.<sup>40</sup> Cortisol has been repeatedly shown to peak in the morning and to fall in the evening,<sup>2, 5, 7, 34</sup> as was also shown in our study. These rhythmical physiological events may explain why  $IgA_{sr}$  and  $Saliva_{sr}$  readings were lower in the morning than in the evening.

#### Take home message

This study shows that there is a morning circadian lowering of IgA and saliva secretory rate and an increase in cortisol, indicating that athletes should avoid early morning training. This will not be acceptable to elite competitors, whose regimens often require two or even three daily sessions. However, early morning sessions should perhaps be avoided by those returning to training after injury or illness, those close to periods of important competition (which are more associated with underperformance syndrome), and possibly those training at altitude, which itself imposes a degree of immunosuppression.

Swimming did not induce any significant changes in morning and evening salivary IgA concentration and  $IgA_{sr}$  (figs 1 and 2), which supports previous results.<sup>9, 41</sup> In general, exercise increased IgA concentration and decreased  $IgA_{sr}$ , although not significantly.

Despite the non-significant exercise induced changes observed in IgA concentration and secretory rate,  $Saliva_{sr}$ , which was significantly decreased ( $p < 0.001$ ) after exercise, may be the most influential factor in terms of protection against oral pathogens and infections.<sup>20</sup> This can be supported by findings showing that people suffering from xerostomia (dry mouth syndrome) have a substantially increased incidence of oral infections and more pathogenic bacteria in the buccal cavity.<sup>20</sup> These results appear to suggest that submaximal swimming may detrimentally affect the quantity of saliva produced, but not its quality, a finding supported by other studies.<sup>17</sup>

Time of day did not have any significant effect on the magnitude of IgA concentration,  $IgA_{sr}$  and  $Saliva_{sr}$  responses to submaximal swimming exercise. However, it has been reported that the magnitude of immune responses to exercise is independent of the circadian variation in lymphocyte subpopulations.<sup>15</sup> The magnitude of the decrease, although non-significant between morning and evening for both  $IgA_{sr}$  and  $Saliva_{sr}$ , was greater in the morning—that is, when flow rate is low—than the evening—when flow rate is high. This emphasises that it may be more hazardous, in terms of susceptibility to oral infections, to exercise in the morning than the evening. In addition, correlation analysis revealed significant inverse relations (at am and pm) between the baseline values of  $IgA_{sr}$  ( $r = -0.891$ ,  $p < 0.000$  and  $r = -0.840$ ,  $p < 0.000$  respectively) and  $Saliva_{sr}$  ( $r = -0.906$ ,  $p < 0.000$  and  $r = -0.665$ ,  $p < 0.001$  respectively) and their exercise induced response. These results suggest that the responses of  $IgA_{sr}$  and  $Saliva_{sr}$  to exercise are affected by their baseline levels, which in turn are dependent on circadian rhythms.

A portion of the cortisol levels, IgA concentration,  $IgA_{sr}$  and  $Saliva_{sr}$  measured before exercise could be attributed to anticipatory psychological stress.<sup>20</sup> Psychological stress operates as a cofactor in the increase in cortisol level<sup>24, 29, 41</sup> and the decrease in  $Saliva_{sr}$  by sympathetic activation<sup>29</sup> and  $IgA_{sr}$ .<sup>20, 40</sup> Therefore, the baseline values of the variables cannot be considered true stress-free control conditions. This is a limitation of the study.

The results of this study suggest that the optimal time for training—that is, the time of day with the least immunosuppressive effect—is the evening, because basal and post-exercise cortisol levels are low, and the flow rate of saliva/mucosal barrier against oral pathogens is then at its peak (both before and after submaximal swimming). We suggest that, in future exercise and immunoendocrinological studies, data are collected at identical time points.

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#### Authors' affiliations

L Dimitriou, N C C Sharp, Department of Sport Sciences, Brunel University, Osterley Campus, Borough Road Isleworth, Middlesex TW7 5DU, UK

M Doherty, Department of Sport and Exercise Science, University of Luton, Park Square, Luton, Beds LU1 3JU, UK

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#### Note to readers

Since February 2001, we have included colour pictures of sporting activities on the cover of the *British Journal of Sports Medicine*. We hereby solicit your ideas and contributions for future covers of the *British Journal of Sports Medicine*. Original artwork, photographs, and posters may all be considered. We will credit the photographer and athlete(s)/team on the contents page of the issue.

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