

and sometimes overtraining syndrome. While overreaching can be managed in a way that improves athlete performance, overtraining results in a prolonged performance decrement. Thus, professional athletes should be carefully monitored to assess the effects of training on individual well-being and performance, and consequently establish the most appropriate recovery plan. The best way for tailoring recovery of each single player in a team is the evaluation of individual biochemical parameters.^[3] In this regard, several metabolites can be useful as biomarkers for tracking the individual performance, and identifying the individual balance between training and recovery in elite athletes.

Most of biochemical tests have been carried out using blood samples, that are collected in an invasive way, and therefore disliked by most of athletes. Notably, plasma biomarker concentrations have been shown to be highly correlated with the salivary ones, so that changes in salivary analytes concentration reflect changes not only in the oral cavity, but also in distant organs and tissues.^[4–6] Therefore, the use of saliva tests in monitoring hormones, immune markers or oxidative stress, can be regarded as an alternative method to monitor athletes without adopting invasive procedures; hence it can also facilitate the compliance of athletes to be recruited.^[4,7]

Only a limited number of studies have observed young athletes in order to evaluate their behavior and the correlation between physiological biomarkers and stress. Given the young age of athletes, this is crucial for maintaining good health and preventing diseases. This study aimed to carry out saliva testing in teenage water polo athletes for assessing the variability of stress response biomarkers at different times of competitive activity, that is, during an intense training session and during a competition.

2. Materials and methods

2.1. Study cohort

Seventeen Sicilian teenage male water polo players (age 15.6 ± 0.89 years, height 176 ± 7 cm, weight 73.4 ± 9.6 kg, body mass index (BMI) 23.6 ± 2.2 kg/m²) of the team Water Polo CUS UniME participated in this study.

The players followed a training routine of 6 days per week, based on a mesocycle preparation program, as previously reported.^[8]

The study was carried out in accordance with Helsinki declaration and approved by Water Polo CUS UniME team coach and medical staff, and by the Ethics Committee of Polyclinic Hospital University (approval protocol number 4157/2021, date February 8, 2021). Written informed consent was released by the parents of all participants.

2.2. Collection of saliva samples

Saliva collection was performed in 3 different moments: early morning (M, between 7:00 and 9:00 AM), early afternoon (B, 10 minutes before the match), and late afternoon (A, immediately after the match) both on a day of training match (T1) and on a day of friendly match (T2). Saliva samples were collected using Salivette devices Sarstedt (Medical Systems, Genova, Italy) according to manufacturer's instructions.

2.3. Biochemical tests

Total salivary proteins were quantified using the standard Bradford assay.^[9]

Indirect competitive ELISA kits (Abcam, Prodotti Gianni, Milan, Italy) were used for salivary cortisol and testosterone quantification. Briefly, according to the manufacturer's guidelines, 75 μ l standards or samples were added in a 96-well plate. Then, 100 μ l enzyme conjugate were added in each well, and the plate was incubated in an orbital shaker at room temperature for 1 hour. After 5 washes with 400 μ l wash buffer, 200 μ l substrate solution were added to each well, and the plate was incubated in the dark for 30 minute. The enzyme reaction was stopped by pipetting 100 μ l stop solution into each well and the absorbance was read at 450 nm using a microplate reader (Tecan, Italy).

In order to detect secretory immunoglobulin A (s-IgA) a direct ELISA kit (Cloud-Clone Corp., DBA Italia, Segrate, Italy) was used, according to manufacturer's instructions. Briefly, 100 μ l standards or diluted samples were incubated in a 96-well plate for 1 hour at 37°C. After liquid removal from each well, 100 μ l detection reagent A were added to each well, and the plate was incubated for 1 hour at 37°C. After 3 washes with 350 μ l wash buffer, 100 μ l detection reagent B were added to each well, and the plate was incubated for 30 minute at 37°C. After 5 washes, similarly to the previous wash step, 90 μ l substrate solution were added in each well, and the plate was incubated in the dark for 15 minute. Then, 50 μ l stop solution were added in each well, and the absorbance was read at 450 nm using a microplate reader (Tecan, Italy).

The determination of advanced oxidation protein products (AOPP) was performed in diluted saliva samples (1:5 in phosphate buffered saline, pH 7.2) by a colorimetric assay using Chloramine T as standard, as previously reported.^[10] Briefly, 200 μ l saliva and chloramine-T standards (0–100 μ mol/l) were added into a 96-well plate. Then, 10 μ l potassium iodide (1.16 μ mol/l) were added to each well, and the plate was incubated for 15 minute. Finally, 20 μ l glacial acetic acid were added to each well to stop the reaction. The absorbance at 340 nm was read after 2 minutes. The concentration of AOPP in the samples was expressed as nmol/ml of chloramine-T equivalents or as nmol of chloramine-T equivalents per mg of salivary proteins.

2.4. Statistical analyses

The numerical data were expressed as means and standard error (SEM). The distribution of examined variables was not found normal distribution, as verified by Kolmogorov–Smirnov test. Then, the nonparametric Kruskal Wallis test and Mann–Whitney *U* test were used for comparison of the 3 data sets and the two-by-two comparisons between groups.

The Spearman correlation test was applied to determine the existence of any significant interdependence between the examined variables.

Statistical analyses were performed using Graph Pad statistical software. A *P* value < .05 was considered statistically significant.

3. Results

Salivary protein concentrations measured on day T1 were significantly lower than those on day T2 (range 0.14–1.12 mg/ml vs 0.08–2.74 mg/ml, *P* = .003). On day T1, mean protein concentrations measured before match (B) and after match (A) were about 50% lower than those at morning (M) (T1-M vs T1-B *P* = .004; T1-M vs T1-A *P* = .006) (Table 1). On day T2, protein concentrations increased, even if not significantly, from morning (T2-M) to the time before match (T2-B) and after match (T2-A) (Table 1).

Table 1
Exercise intensity-dependent variations of salivary total protein levels in teenage male water polo players.

Time of the day	Total salivary proteins (mg/ml)	
	T1	T2
M	1.53 ± 0.14	0.41 ± 0.08 [†]
B	0.85 ± 0.11*	0.58 ± 0.1
A	0.76 ± 0.1*	0.48 ± 0.2

Values are shown as means ± SEM.

A = after match, B = before match, M = morning, T1 = training match day, T2 = friendly match day.

* $P < .01$, significant difference in comparison with T1-M.

[†] $P < .0001$, significant difference in comparison with T1-M.

Total salivary protein concentrations were subsequently used to normalize concentrations of salivary cortisol, testosterone, s-IgA and AOPP, in order to have a more accurate estimation of changes in the concentration of performance and stress biomarkers investigated in this study.

The lowest cortisol concentrations were measured in T1-A and were significantly different from those in T1-M (Fig. 1A) ($P = .025$), but not in T1-B (Fig. 1A). On the contrary, cortisol concentrations gradually increased from T2-M, reaching the highest values in T2-A (not significant) (Fig. 1A). Instead, significant differences were found when comparing T1-M vs T2-M cortisol concentrations ($P = .035$) and T1-A vs T2-A ($P = .045$). These trends were confirmed even after normalization of cortisol concentrations against total protein salivary content. However, statistically significant differences were not confirmed (Fig. 1B).

The highest mean testosterone concentrations were measured in T1-M and T2-M, and they were about 4.5-fold and 2.5-fold higher than those measured in T1-A ($P = .013$) and T2-A ($P = .002$), respectively. T2-A testosterone levels were higher than T1-A ones (Fig. 2A), but this difference only tended to a statistically significant value ($P = .09$).

The decreasing trend of testosterone concentrations throughout both days was confirmed after testosterone level normalization against total salivary proteins only for day T1 (Fig. 2B). However, no significant differences were found when comparing testosterone concentrations measured at different times of the same day. Instead, significant differences were observed between the 2 days, since T2-M testosterone/proteins values were significantly higher than T1-M ones, while the difference between T2-A and T1-A ratios tended to be statistically significant ($P = .09$).

No significant differences were found when comparing free testosterone/cortisol ratio (T:C) ratio variations on day T1 (T:C T1-A vs T1-M 0.02 ± 0.0031 vs 0.057 ± 0.015) and day T2 (T:C T2-A vs T2-M 0.053 ± 0.021 vs 0.107 ± 0.031), although on both days the T:C ratio decreased by 2-fold from morning to the time after match, indicating the occurrence of a catabolic response in athletes following exercise.

The mean salivary s-IgA concentrations measured on day T2 were higher than those in T1 (53-880 vs 24-460 $\mu\text{g/ml}$), with a significant difference observed between T2-A and T1-A ($P = .048$). An increasing trend was observed throughout the day on both sampling days. Indeed, T1-A and T2-A s-IgA concentrations were 1.8-fold and 3.5-fold higher than T1-M and T2-M ($P = .041$), respectively (Table 2). This trend was confirmed even after normalization of s-IgA concentrations against total salivary proteins. However, the differences between values measured at

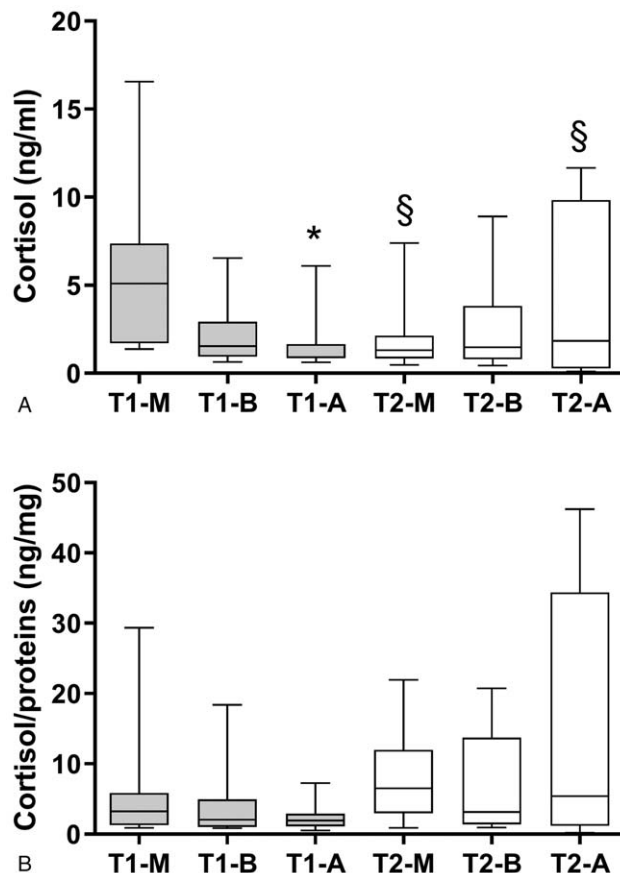


Figure 1. Exercise intensity-dependent variations of salivary cortisol levels in teenage male water polo players. Cortisol concentrations (means ± SEM) are shown as either total or normalized against total salivary proteins. A = after match, B = before match, M = morning, T1 = training match day, T2 = friendly match day. * $P < .05$, significant difference in comparison with T1-M; § $P < .05$, significant difference in comparison with T1-A.

morning and after match increased. In particular, T1-A and T2-A s-IgA concentrations were 2.8-fold and 4.2-fold higher than T1-M and T2-M, respectively (Table 2). Notably, normalized s-IgA against proteins on day T2 were significantly higher than those recorded on day T1 at morning ($P = .008$) (Table 2), whereas the difference only tended to a statistically significant value when comparing T2-A with T1-A ($P = .08$).

AOPP increased from M to A on both T1 and T2 days. T1-A AOPP concentrations were significantly higher than those in T1-M ($P = .005$) (Table 3). T2-B AOPP concentrations were significantly higher than those in T2-M ($P = .02$) and T2-A ($P = .047$). T2-A concentrations were higher than those in T2-M, even if not significantly (Table 3).

T2-M and T2-B AOPP concentrations were significantly higher than T1-M ($P = .019$) and T1-B ($P < .001$), respectively, whereas only a difference tending to statistical significance was found when comparing T2-A AOPP concentrations with T1-A ones ($P = .08$).

The normalization of AOPP levels against salivary proteins confirmed the increasing trend observed on day T1, and the significant difference between T1-A AOPP concentrations and T1-M ($P = .002$). Instead, on day T2, AOPP/proteins ratio a continuously increased from M to A, even if not significant

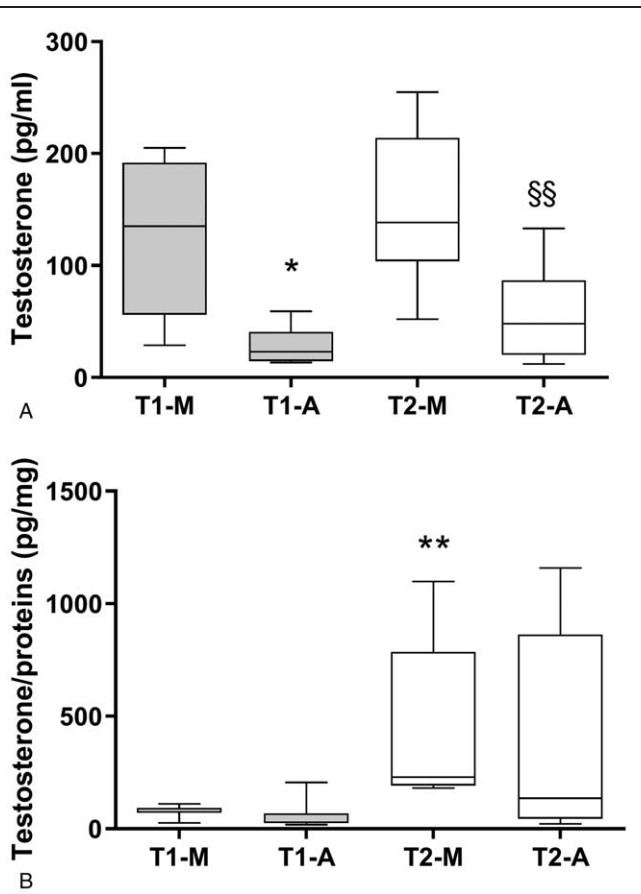


Figure 2. Exercise-dependent intensity variations of salivary testosterone levels in teenage male water polo players. Testosterone levels (means ± SEM) are shown as either total or normalized against total salivary proteins. A = after match, B = before match, M = morning, T1 = training match day, T2 = friendly match day. **P* < .05, †*P* < .01, significant difference in comparison with T1-M; ‡*P* < .01, significant difference in comparison with T2-M.

differences were found. Only T2-B ratio tended to be significantly higher than T2-M ratio (*P* = .08). Notably, AOPP/proteins ratios measured on day T2 were significantly higher than those recorded on day T1 at any time of the day (T2-M vs T1-M *P* < .001; T2-B vs T1-B *P* < .001; T2-A vs T1-A *P* = .003).

A positive correlation was found between Cortisol/Proteins and Testosterone (*r* = 0.888, *P* = .003), as well as IgA/Proteins (*r* = 0.857, *P* = .014) on day T1, and between Cortisol/Proteins and AOPP on day T2 (*r* = 0.735, *P* < .001).

4. Discussion

This work was aimed to evaluate the stress response in water polo teenage elite athletes in a training session or in a competitive session. We measured changes of multiple biomarkers, namely cortisol, testosterone, s-IgA, and AOPP, that are useful to assess exercise performance, immune activation and oxidative stress, respectively. Results obtained provide useful information for this age group, that has been so far examined by very few studies, addressing, however, other sports.

Here we observed a high variability in the concentrations of different biomarkers depending on activity session. Our results confirm previous observations indicating an elevation of total protein concentration along with the increase of workout intensity, that is, during the friendly match, and a decrease during a less intense training session.^[11]

Cortisol monitoring in sports is useful to assess the stress response to physical efforts, and its concentration is influenced by several factors such as sex, age, population, circadian rhythm, hydration state, and also exercise, especially if intense.^[4,12,13] We observed an increase in salivary cortisol levels from pre- to post-match, in line with concentrations and trend observed in young male volleyball, basketball, and soccer players,^[11,14-17] and a higher cortisol increase after friendly match than training match, given the different stress conditions to which the athletes were subjected. Indeed, match-related physical effort leads to activation of hypothalamic-pituitary axis, with subsequent elevation of cortisol release. This increase, associated with a decrease in testosterone, represents the activation of catabolic process necessary for the supply of energy.^[18] Moreover, the cortisol levels measured before the friendly match were higher than those measured before the training match, suggesting that an anticipatory increase of salivary cortisol occurs before sport competition, and is characteristic of athletes under psychosocial and physical stress conditions. These data are in agreement with previously reported ones,^[19] who highlighted also a positive role of cortisol increase in cognitive processes.

We also observed that testosterone levels, despite decreasing throughout both days, remained significantly higher in T2 compared to T1. These findings confirm previously reported observations. In fact, relatively short training sessions produce an increase in hormone levels, whereas longer sessions, or at very high intensity, lead to a decrease in its concentration.^[18] The pre-match testosterone level observed in T2 may be related with preparatory and aggressive behavior increasing vigor leading to a dominant performance.

The hormonal variability observed in many studies could be attributed to physical/physiological factors related to competitive

Table 2
Exercise intensity-dependent variations of salivary sIgA levels in teenage male water polo players.

Time of the day	T1		T2	
	s-IgA (μg/ml)	s-IgA/proteins (μg/mg)	s-IgA (μg/ml)	s-IgA/proteins (μg/mg)
M	38.5 ± 5.3	25.0 ± 7.8	56.0 ± 12.3	293.0 ± 83.3 *
A	54.0 ± 7.7	71.0 ± 17.3	196.0 ± 47.7 †,‡	1073.0 ± 438.2

Values are shown as means ± SEM.
A = after match, M = morning, T1 = training match day, T2 = friendly match day.
**P* < .01, significant difference in comparison with T1-M.
†*P* < .05, significant difference in comparison with T2-M.
‡*P* < .05, significant difference in comparison with T1-A.

Table 3
Exercise intensity-dependent variations of salivary AOPP levels in teenage male water polo players.

Time of the day	T1		T2	
	AOPP (nmol/ml)	AOPP/proteins (nmol/mg)	AOPP (nmol/ml)	AOPP/proteins (nmol/mg)
M	3.68 ± 0.7	2.84 ± 0.56	7.94 ± 1.68 ^{*,§}	20.92 ± 11.8 [‡]
B	5.83 ± 1	12.8 ± 4.09	31.47 ± 5.65	56.0 ± 10.86
A	8.78 ± 1.2 [†]	15.98 ± 3.16 [†]	12.09 ± 1.28 [§]	78.05 ± 24.2 [¶]

Values are shown as means ± SEM.

A = after match, B = before match, M = morning, T1 = training match day, T2 = friendly match day, and different times of the day.

* $P < .05$, significant difference in comparison with T1-M.

† $P < .01$, significant difference in comparison with T1-M.

‡ $P < .001$, significant difference in comparison with T1-M.

§ $P < .05$, significant difference in comparison with T2-B.

|| $P < .001$, significant difference in comparison with T1-B.

¶ $P < .01$, significant difference in comparison with T1-A.

level, gender, match-play activity profile, and athletic performance, that is, elite athletes had higher testosterone but lower cortisol levels, whereas amateurs vice versa.^[20,21] In water polo playing time may greatly vary from athlete to athlete, so that game time, player role and individual physiological demand can differently affect stress response.^[22]

Hormonal response to exercise is a useful indicator of overreaching or overtraining status. In this regard, the T:C gives an indication of the anabolic/catabolic balance in response to training.^[23] A prolonged decrease of this ratio reflects an increased proteolysis and a reduced protein synthesis. A 30% T:C ratio decrease indicates insufficient recovery, while a value of 0.35×10^{-3} is considered the threshold for overtraining.^[23] Since salivary T:C ratio correlates well with serum one,^[24] it can be used for monitoring hormonal response in elite athletes. Although T:C ratios in teenage water polo athletes decreased after match on both days, they were far from the established overreaching threshold.^[23] Surprisingly, T:C ratios on day T2 were higher than on day T1, due to the increase of testosterone at morning in anticipation of impending competitive match, as reported by several authors.^[25] The significant positive correlation between Cortisol/Proteins and Testosterone is in line with that reported in soccer players.^[24]

It is known that acute exercise also produces changes in immunological response. sIgA are considered a good indicator of mucosal innate immunity, providing the first line of defense against infections of the upper respiratory tract.^[26] Several reports addressing the variability of salivary sIgA concentrations in young athletes (under 17) involved in team sport activities, showed that sIgA levels are influenced by seasonal changes, match importance, and exercise intensity/kind of effort.^[14,27-30] Notably, competitive sport induces a completely different adaptive response to stress in comparison with sedentary individuals.^[31]

We observed a significant increase of sIgA levels after the friendly match, in line with findings on endurance athletes after a high intensity interval session.^[32] Notably, we first report that salivary sIgA/proteins ratio is positively correlated with cortisol/proteins. Given the established correlation between salivary and serum sIgA as well as cortisol,^[4] our findings are likely in line with previously reported correlation between serum sIgA and cortisol.^[29] Indeed, stress-related epinephrine and cortisol work along the pathway of beta2-adrenergic receptors, present at high density on B cells, thus increasing sIgA levels.

While regular/moderate exercise or recreational sport activity significantly improve redox parameters and provide many health

benefits, intense exercise/competitive sport can generate an excess of oxidative stress with consequent damages to the organism.^[33]

We assessed oxidative stress using salivary AOPP as biomarker, since salivary protein oxidation is the first line of defense against free radicals in oral tissues.^[34] Reported concentrations of salivary AOPP in resting Caucasian young males (<25 nmol/mg protein) show a high inter- and intra-individual variability, about 60% and 45%, respectively.^[10,35] Our observations indicated that salivary AOPP levels increased after exercise and were significantly higher on day T2 than day T1. Given the reported correlation between salivary and serum AOPP levels,^[35] these findings agree with previous observations in serum samples of water polo players.^[8,36]

AOPP levels were positively correlated with the ratio Cortisol/Proteins on day T2. No literature findings are available on cortisol-AOPP correlation. However, cortisol is known to increase concomitantly with increased oxidative stress after acute exercise.^[37,38]

As a general consideration, it should be highlighted that the variation trends of the redox markers investigated in different studies are often not superimposable to each other because of different type of physical activity, age, gender, healthy nutrition, or supplementation of antioxidants,^[39] as well as inter- and intra-individual variability.

In conclusion, we found that the salivary cortisol, testosterone, sIgA, and AOPP, are affected by different types of activities, such a training or competitive session. In particular, we observed an increase of cortisol, IgA e AOPP levels during T2 compared to T1, which may be attributed to a high psychological stress of participating in a competition and an elevation of the training load during the game. In relation to testosterone, we observed a decrease on both days, but it still remained significantly higher in T2 than in T1 since it is implicated in competitive behavior.

Sports competition has been proposed as a situation that causes anxiety, including both physiological and psychological stressors.^[40] and the hormonal response is a necessary resource for improving physical activity, memory, learning, and emotions, which represent basic capacities for good performance.

The results showed that performance in competitions induces a stress response in athletes, with an anticipatory response of cortisol and testosterone before competition. This is why competitive situation usually manifests itself even before the competitive activity and begins to stimulate an intense response from the participants. Cortisol performs a homeostatic function by regulating other stress-sensitive systems, which can also affect

the secretion of sIgA. As showed in our study, an acute exercise stimulates the increase in sIgA secretion, that may be related to the improvement of immunity. However, it would be interesting to evaluate if the mucosal immune defenses remain after a few days from training.

Moreover, we first report that salivary AOPP levels are a good marker for assessing redox changes in response to low intensity or high intensity competitive activity.

This work also confirms that saliva testing is a noninvasive way for monitoring the stress response of young elite athletes to acute exercise. Results obtained can be employed for tailoring recovery strategies in the perspective of following competitions and improve the individual fitness of athletes.

Limitations of this study were: the absence of subjective measures of athlete well-being, that is, Profile of Mood States (POMS) and Perceived Stress Scale (PSS), to assess the psychological impact of stress/fatigue^[41]; the small number of examined subjects, likely amplifying inter-individual differences; difficulties of data comparison due to few studies performing saliva tests in young elite athletes, and the lack of data on water polo athletes.

In view of these considerations, further experimental research is needed to address these limitations.

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