Interaction between stress and immunity during a week leading to competition in young athletes

by

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Submitted in partial fulfillment of the requirements for the degree of
Master of Science in Applied Health Sciences
(Kinesiology)

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Abstract

This study examined changes in the salivary concentrations of immunoglobulin A (sIgA), cortisol (sC), testosterone (sT) and testosterone-to-cortisol ratio (T/C) in 23 competitive swimmers, 11-15 years old, during a week leading to competition as compared to a control (non-competitive) week. Results showed no effect of week or day, and no significant week-by-day interaction for sIgA, sC and T/C. In contrast, sT significantly decreased during the week of competition, along with a 7%, non-significant decrease in the weekly T/C. The latter suggests that the swimmers were in a catabolic state due to their training, but this did not have a negative effect on their performance Since sC did not change over the two weeks and according to the sport anxiety scale, competition stress was relatively low in these peri-pubertal athletes, it is concluded that in the absence of high cortisol levels mucosal immunity is unaffected in young athletes prior to competition.

Acknowledgements

I would like to thank my supervisor, Dr. Nota Klentrou, for providing me with the support, guidance and encouragement I needed in my graduate studies and for standing by me during the good and not so good times of this research. I would also like to thank the members of my advisory committee, Drs. Bareket Falk and Brian Timmons, for their guidance and support. I would like to acknowledge the most valuable contribution of Dr. Cameron Muir for taking the time out of his schedule to help make this research what it has become. Many thanks go out to my lab mates, Colin Russell and Izabella Ludwa for helping me to collect the data and recruit the participants. Most importantly, an important thank you goes to the parents and swimmers who participated in the study. And finally, a thank you to my parents and sister for all their support and for helping me stay on track.

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List of Abbreviations

%CV: Percent coefficient of variation

sC: Salivary cortisol

sT: salivary Testosterone

T/C ratio: Testosterone/Cortisol ratio

sIgA: Salivary immunoglobulin A

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Chapter 1: Introduction

1.1 Rationale

Elite, young swimmers are characterized by a heavy training load. This may play a role in the response of their mucosal immunity since there is some evidence in the literature that long periods of training can lead to immunosuppression in adult swimmers (Fricker et al. 1999, Gleeson et al. 1995a, Gleeson et al. 1995b, Tharp et al. 1990). There is also considerable inconsistency in the literature; immune markers such as salivary Immunoglobulin A (sIgA) have been shown to decrease in response to prolonged training in swimmers (Gleeson et al 1999), whereas another study found no significant changes in sIgA after a 15-week training (Pyne et al. 2000).

Furthermore, competition events have been shown to cause an increased stress reflected by higher cortisol levels (Filaire et al 2001b, Moreira et al. 2013), and this may have an additional immunosuppressive effect. However, although the relationship between immune markers and cortisol has been studied extensively in adult populations a clear association has not been established due to conflicting results. For example, many studies have shown that there is no association between immune markers such as sIgA and cortisol levels, suggesting that stress does not affect immunity directly (Cieslak et al. 2003, Filaire et al. 2004). Other studies have reported a negative effect of stress on immunity in adults (Cheng-Shiun He et al. 2010, Gleeson et al. 1995, Hucklebridge et al 1998). However, all these studies have measured the levels of salivary cortisol, and other stress hormones, without taking into account the variability of these hormones. The day-

to-day variability of stress hormones (as measured by the % coefficient of variation) may be a better indication of adaptability than a single measurement (Carré et al. 2011, Cieslak et al. 2011). People with high hormonal variability considered more adaptive and able to cope with situations such as stress. Finally, Gleeson et al. (1995b) reported a negative relationship between perceived stress and immunity in elite adult swimmers but did not measure cortisol or other biological markers of stress. In addition, there are no relevant studies that examine the potential chronic effect of stress the days leading to a competition in young or adult athletes. Therefore, the aim of this study was to determine whether the combination of training and competition stress have an effect on the salivary concentration and variability of stress hormones resulting in compromised salivary concentrations of sIgA in young swimmers during a week leading to competition, as compared to a control (non-competitive) week.

1.2 Objectives

- Examine changes in the salivary concentration of sIgA, cortisol (sC), testosterone (sT) and testosterone-to-cortisol ratio (T/C) in young swimmers during a week leading to competition as compared to a control (non-competitive) week.
- Investigate whether the changes and/or the variability (measured by % coefficient of variation) of the stress hormones between the two weeks is associated with changes in mucosal immunity.

1.3 Hypotheses

- The sC would gradually increase while the sT and the T/C ratio would decrease during the week leading to competition as compared to the control week.
- Mucosal immunity, as determined by sIgA levels, would decrease during the competition week, and this change will be associated with the changes and/or increased variability of stress hormones.

Chapter 2: Review of Literature

2.1 The Immune System

The body's ability to protect itself from bacteria, viruses, and other disease-causing entities is known as immunity (Silverthorn et al. 2006). The skin enzymes such as lysozyme, and mucus constitute the first line of defense. These factors either have antimicrobial function or prevent the attachment of pathogens. In order to have an infection, potential bacteria or viruses must penetrate all those barriers (Silverthorn et al. 2006, Delves et al. 2000). Once pathogens break through that line of defense, the internal immune response takes over (Silverthorn et al. 2006). The immune response can be non-specific (innate immunity), or acquired and specific to the pathogen (adaptive immunity) (Silverthorn et al. 2006).

2.2 Immunoglobulins

Immunoglobulins are glycoprotein molecules secreted by plasma cells. There are five classes of immunoglobulins, M, G, A, E and D, with structural and functional differences (Silverthorn et al. 2006, Späth 1999).

Immunoglobulin A (IgA) is the second most abundant antibody in serum after IgG, while at mucosal surfaces such as saliva, IgA exhibits higher levels compared to IgG (Schroeder et al. 2010). IgA reduces colonization and plays a crucial role in the protection of mucosal surfaces from toxins, bacteria, and viruses (Späth 1999, Schroeder

et al. 2010). It acts in two ways against pathogens: a) direct neutralization or b) prevention of binding to the mucosal surface (Schroeder et al. 2010).

2.3 Mucosal Immunity

Mucosal immunity consists of several cellular and non-cellular components (Mayer 2003). The mucosal immune system is composed of gut-associated lymphoid tissue (GALT), urogenital tracts, lacrimal glands, lactating mammary glands, bronchus-associated lymphoid tissue (BALT), salivary glands, and nasal associated lymphoid tissue (NALT) (Gleeson 2000b). Mucosal immunity, in conjunction with the non-specific immune response, constitutes the first line of defense against foreign substances (Gleeson et al. 2000c).

2.3.1 Saliva Composition

Saliva is the colorless fluid that exists in the oral cavity and plays an important role to oral health. Parotid, submandibular, sublingual, and minor salivary glands are responsible for salivary production. Water is the most abundant component of saliva (>99%). Many inorganic compounds such as (sodium, calcium, potassium, chloride, magnesium, phosphate, and bicarbonate) are also found in saliva. Organic constituents comprise urea, uric acid, creatinine, ascorbic acid, serum albumin, and hormones such as cortisol and testosterone (Chicharro et al. 1998). Saliva is highly correlated with immunological reactions due to its many components with antimicrobial and antiviral functions including IgA that is the largest immunologic component of saliva. (Del Vigna et al. 2008).

Consequently, salivary IgA (sIgA) is critical in order to maintain mucosal immunity while salivary IgM plays an important role when sIgA levels are very low (Gleeson et al. 2000c). People with sIgA deficiency are more susceptible to upper respiratory tract infection (URTI) (Gleeson 2000b).

Lysozyme, lactoferrin, and peroxidase are also saliva compounds that contribute to the inhibition of bacterial adherence and provide fungicidal and antiviral functions (Del Vigna et al. 2008). Salivary proteins such as statherins, mucins, cystatins and histatins are also correlated with a bunch of physiological and immunological functions. Typically some of the saliva components are found also in serum with the percentage of total proteins in saliva being much lower compared with that of serum (Esser et al. 2008).

2.4 Relationship between secretory Immunoglobulin A and URTI

Many studies tried to determine if a correlation exists between upper respiratory tract infections (URTI) and altered levels of sIgA during training in athletes. Elite swimmers exhibited low levels of sIgA and high URTI episodes within a seven-month period of training (Gleeson et al. 1999). However, two subsequent studies have also suggested that no relationship exists between sIgA and URTI incidence in swimmers (Pyne et al. 2000, Gleeson et al. 2000a).

In agreement with these results in swimmers, Tsai et al. (2009) found no correlation between decreased sIgA levels and URTI in taekwondo athletes. Likewise Nakamura et al. (2006) showed that there is no significant relationship between decreased sIgA and frequency of URTI symptoms in soccer players. However, this study found

decreased saliva flow rate and sIgA secretion rate three days before the appearance of URTI symptoms. This relationship has been examined mainly in adults, with little evidence regarding this correlation in children. According to Cieslak et al. (2003), children that are habitually active have higher sIgA levels and reduced incidence of URTI compared to children that are inactive.

2. 5 Exercise Training and sIgA

Cross-sectional and longitudinal studies have investigated the effects of long-term exercise on salivary immunoglobulins. The results have shown that potential decreases in mucosal immunity are evident after a few days of intense training but longer periods of intense training (>3 months) lead to significant immunosuppression (Gleeson 2000b). The majority of the studies support that sIgA decreases as a result of prolonged, intense training (Gleeson et al. 1995, Gleeson et al. 1999, Cheng Shiun He et al. 2010, Fricker et al. 1999, Mackinnon et al. 1993, Tharp et al. 1990, Tomasi et al. 1982, Tsai et al. 2011). Others have showed that sIgA increases after moderate exercise training (Tharp et al. 1991, Gleeson et al. 2000a, Klentrou et al. 2002). The study by Gleeson et al. (2000a) provides information regarding the response of sIgM and sIgG to long-term training. Specifically, sIgM presents small increments whereas sIgG remains unchanged during a 12-week training cycle in elite swimmers (Gleeson et al. 2000a).

There are a few studies that tried to examine sIgA levels in children in response to chronic exercise. A study showed that young tennis athletes do not exhibit significant changes in sIgA levels within a 12-week training period (Novas et al. 2003). In contrast,

Tharp et al. (1991) indicated that pre- and post-pubescent basketball players exhibit increased sIgA levels prior to practice and prior to competition over two months of saliva collection.

The conflicting results in the literature lead us to the conclusion that the chronic effects of exercise training on mucosal immunity are not well established while there is little evidence regarding the response of mucosal immunity in young athletes leading to competition.

2.6 Markers of Biological Stress

2.6.1 Cortisol

The activation of the hypothalamic-pituitary-adrenal (HPA) axis is responsible for cortisol secretion. The hypothalamus secretes corticotropin-releasing hormone (CRH) which promotes the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Silverthorn et al. 2006, Tortora et al. 2009). Cortisol is released when ACTH acts with adrenal cortex. Once cortisol is released, it inhibits ACTH and CRH secretion (Silverthorn et al. 2006, Tortora et al. 2009).

Cortisol affects glucose formation through gluconeogenesis when liver cells convert amino acids to glucose, which other cells can use for ATP production (Silverthorn at al. 2006,Tortora et al. 2009). The breakdown of protein promoted by cortisol, provides a substrate for gluconeogenesis (Silverthorn et al. 2006, Tortora et al. 2009). Cortisol stimulates lipolysis through the breakdown of triglycerides and the release of fatty acids (Silverthorn et al. 2006, Tortora et al. 2009). Cortisol also acts as an

anti-inflammatory agent by suppressing the leukocyte response (Tortora et al 2009). Another characteristic of cortisol is that it affects brain function. High levels or lack of cortisol causes mood alterations and memory disturbances (Silverthorn et al. 2006).

Cortisol levels seem to be influenced by the event and the type of competition. According to Filaire et al 2001, salivary cortisol increases the days of a major competition in judo athletes while the same phenomenon observed in volleyball players prior to a significant volleyball match (Moreira et al 2013). In agreement with the previous results, another study suggests that salivary cortisol increases in basketball players before competition (He et al. 2010).

2.6.2 Testosterone

Testosterone is the most abundant male steroid hormone. Leydig cells, which are found in the interstitial tissue in the testes, are responsible for testosterone's production whereas minor amounts are secreted from the prostate, skin, and liver (Loebel and Kraemer 1998). Testosterone levels are high in men under the age of 30 while decreases by the sixth decade of life (Loebel and Kraemer 1998). Prepubertal males exhibit low and stable testosterone values from six to nine years of age, whereas from the age of nine to ten testosterone increases depending on pubertal development (Di Luigi et al. 2005). Cholesterol plays the most important role to testosterone's synthesis. Half of the testosterone that is produced in the testes is derived from de novo cholesterol synthesis and the most critical phase in the synthesis of testosterone is the conversion of cholesterol to pregnenolone (Loebel and Kraemer 1998). The next step is the conversion of

pregnenolone through two pathways resulting in dehydroepiandrosterone (DHEA) and androstenedione that convert to testosterone.

Testosterone is a sex-related hormone that carries many anabolic properties and developmental phases during life (Loebel and Kraemer 1998). Hair growth, production of secretory proteins, synthesis of erythropoietin, bone development, muscle protein synthesis, and secretion of growth hormone are mediated by testosterone (Loebel and Kraemer 1998). Contrary to cortisol's function, testosterone inhibits the breakdown of muscle glycogen whereas displace the glucocorticoid by attachment to its receptor (Loebel and Kraemer 1998). Those two properties of testosterone play an important role to the retention of muscle protein.

In contrast to cortisol, testosterone is not well examined prior to a significant competition. Nevertheless, evidence from one study shows that salivary testosterone does not significantly changes in adult athletes in response to a significant competition, as compared with a resting day (Filaire et al 2001). Maso et al (2004) showed that overtraining is associated with decreased testosterone in rugby players. However, no evidence exists to date regarding the response of testosterone during a training period in adolescent athletes.

2.6.3 Testosterone-to-Cortisol Ratio (T/C*)*

The relationship that exists between testosterone and cortisol during exercise has been examined in the literature as an indicator of the anabolic/catabolic balance of the body that has been studied in relation to athletic performance (Lac et al. 2000, Passelergue and

Lac 1999). This ratio is considered to reflect states of anabolism when it is high and states of catabolism when it drops (Filaire et al. 2001, Lac et al. 2000).

Most of the studies agree that except the type and the duration of exercise, mood and individual variability influence the T/C ratio. Di Luigi et al. (2005) reported elevated salivary T/C ratio following a 90 min moderate to high intensity training session in pubescent soccer players. The same study concluded that after training, salivary testosterone was increased in late pubertal stages whereas salivary cortisol was elevated only in earlier pubertal stages (Di Luigi et al. 2005). An increased DHEA/C ratio was reported by Filaire et al. (1998) in female volleyball players during the season. Three years later, the same authors reported that the T/C ratio was decreased throughout a year in soccer players (Filaire et al. 2001). Furthermore, professional soccer players tend to have decreased T/C ratio at the conclusion of a competitive season depending on the intensity and duration of their training (Handziski et al. 2006). However, there are no studies on the T/C ratio in children and adolescent athletes.

2.7 Relationship between stress and immunity in Athletes

Psychological stress is defined as the experience of negative events or the perceptions of distress and negative affect that are associated with the inability to cope with them (Cohen et al. 2001). Psychological stress leads to the activation of the hypothalamus pituitary adrenal axis (Gaab et al. 2005). The immune response in a stressful situation varies among people. The activation of both, the hypothalamus pituitary adrenal (HPA) axis and the sympathetic nervous system (SNS) is critical for the the immune response.

Cortisol and catecholamines are the end-products of those two pathways (Herbert et al. 1993). Immune cells that participate in the primary and secondary response have receptors specifically for glucocorticoids and catecholamines which both play an important role in immunoregulation (Cohen et al. 2001, Herbert et al. 1993).

Salivary cortisol (sC) has been extensively examined mostly in adults and adolescents in order to determine potential correlations and interactions with immunity with little evidence regarding this matter in children. According to Gleeson et al (1995) high levels of stress are associated with an increased infection rate and low levels of sIgM. Similarly with the previous study, an inverse correlation was shown between sC and mucosal immunity in basketball players (Cheng-Shiun He et al. 2010). The results of this study indicated that during a basketball season, players exhibit increased levels of salivary cortisol and decreased secretion rates and concentration of sIgA (Cheng-Shiun He et al. 2010). In agreement, Hucklebridge et al. (1998) found a correlation between decreased sIgA levels and increased levels of cortisol.

On the other hand, Filaire et al (2004) found no significant association between sIgA and cortisol levels in young children. In addition, Cieslak et al (2003) suggested that in contrast with physical activity, stress does not affect secretory immunity in children. Results of a recent study have shown that sIgA levels increase or decrease independent of cortisol activity in adult athletes prior to competition (Moreira et al. 2013). Furthermore, the relationship that may exist between sIgA and sC is influenced by multiple factors such as type of sport, individual variability, etc (Cheng-Shiun He et al. 2010). The nature of this correlation in children, especially athletes needs further research.

Chapter 3: Methods

3.1 Participants

This study and all related procedures received ethical clearance from the Brock University Research Ethics Board. After receiving permission from parents and coaches, athletes were recruited from swimming clubs across Southwest Ontario. Thirty 11-15 years old, competitive swimmers were invited to participate in this study. Only swimmers who did not receive a flu shot in the preceding 12 months were invited to participate in the study.

3.2 Study Design

The study was conducted between the end of November and mid-January, to avoid seasonal variations in the outcome measures. A small group of researchers from Brock University met with the young athletes and their parents one week prior to the first experimental week. During this meeting, the participants were provided with a detailed description of the study. A consent form was then completed and signed by the parents of the athletes who agreed to participate in the study (n=38).

Subsequently, anthropometric measures of body mass (kg), height (cm) and relative body fat (%BF) were taken, followed by the completion of a questionnaire package. The questionnaire package included information concerning medical history and sexual maturity. A health log was then handed out to each participant to record URTI

symptoms during each of the two weeks of the study. All questionnaires were completed by the swimmers.

The same procedure was then followed during two non-consecutive weeks: one control, non-competitive week, and one week leading to and including an important competition. To ensure that there was no cross-contamination in the hormonal levels from one week to the next, the two experimental weeks were at least one week apart. To ensure that the competition was similarly important for all swimmers, the competition was chosen so that all swimmers had to achieve specific qualifying standards to advance to the next competitive level. Participants were provided with salivette swabs in order to self-collect one resting saliva sample every morning upon awakening. After the collection, they were instructed to store the saliva sample in -20^{o}C until practice time when they were asked to submit the morning sample to the research team. For both weeks, collection started on the Sunday and continued for 7 days until the Saturday. The competitive week included 2 days of competition (Friday and Saturday). The day before the competition (day 5), swimmers were asked to fill the Sport Anxiety Scale-2 (SAS-2) developed by Smith et al. (2006).

To ensure consistency and account for diurnal fluctuation in hormones, all saliva samples were obtained in the morning upon awakening. Both sC and sT are characterized by a standard fluctuation during the day. Cortisol exhibits peak levels in the morning while it drops in late afternoon (Gröschl et al. 2003, Horrock et al. 1990, Kiess et al. 1995, Rosmalen et al. 2005, Price et al. 1983, Pruessner et al. 1997). Salivary cortisol in children fluctuates in the morning from 3.3 to 26.6 nmol/L while it decreases in the evening (0 to 7.1 nmol/L) (Price et al. 1983, Törnhage 2002). Rosmalen et al. (2005)

provides slightly different values for children. Morning salivary cortisol levels are around 15.38 ± 6.56 nmol/L while evening values are decreased to 1.95 ± 1.33 nmol/L. Salivary cortisol peaks 30-45 minutes after awakening independently of the time of awakening or the total time slept (Pruessner et al. 1997, Rosmalen et al. 2005). One more study verifies the descending pattern that cortisol follows throughout the day in children. According to Gröschl et al. (2003) morning values of sC are 24.7 ± 8.5 nmol/L, whereas at noon it drops to 8.0 ± 4.0 nmol/L. In the evening, sC exhibits the lowest values (1.7 ± 1.4 nmol/L). Generally, testosterone is also characterized by a diurnal rhythmicity with peak concentrations in the morning and decreased levels in the evening (Dabbs 1990, Hayes et al. 2010, Plymate et al 1989, Riad-Fahmy et al. 1983). Similarly, sIgA presents higher levels in the morning than in the afternoon (Dimitriou et al. 2002, Miletic et al. 1996). Passali et al (1988) have shown that sIgA concentration tends to be high in the morning but from 1:00 pm starts an ascending tension that peaks at 4 a.m.

Compliance to daily saliva collection was 75% for the control week, and 80% for the competition week. While the overall compliance was high, the missed days did affect which data were used in the analysis. Thus, only data from participants who had provided at least 5 samples for both weeks were included in the analysis (n=23).

Coaches were asked to provide basic training information and the weekly training schedule for both weeks. During the control week, the swimmers trained 14-19 hours/week (12-16 hours of swimming and 3-4 hours of land/weight training). During the experimental/competition week, the swimmers trained 8.5-9.5 hours/week (6.5-7.5 hours of swimming and 1-2 hours of land/weight training). The training volume (defined as hours per week) was also reduced during the competitive week by an average of 25%

amongst the different groups/teams. Performance data were obtained from the official results, and the difference between seeding and final times were used to quantify each swimmer's performance outcome.

3.3 Experimental Measurements

3.3.1 Anthropometric Measurements and questionnaires

Body mass (kg) and height (cm) were assessed using a weight scale and a stadiometer, respectively. Relative body fat (%BF) was estimated using skinfold thickness (mm) assessed at two sites, the arm and upper back (triceps and subscapular), as previously described (Slaughter et al. 1988). The same investigator completed all anthropometric measures for all participants.

Sexual maturity was self-assessed according to pubic hair development (T_{PH}), as defined by Tanner (Tanner 1962; Taylor et al. 2001). Tanner staging is a well-accepted method to assess sexual maturity in pediatric samples (Di Luigi et al. 2005, Rosmalen et al. 2005). Female participants were also asked to indicate whether or not they had reached menarche, and if so at what age.

Competition anxiety was assessed using the Sport Anxiety Scale 2 (SAS-2), which is a multidimensional measure of cognitive and somatic trait anxiety for children in sport performance settings (Smith et al. 2006).

The frequency of URTI during each week was determined using a daily health log as previously described (Corbett et al. 2010; Nieman et al. 1998). URTIs were recorded in order to: a) identify those swimmers who would have high levels of sIgA due to a

potential upper respiratory tract infection, and b) examine if stress correlates with URTIs independently of sIgA. The log quantifies the frequency and duration (number of days) of URTI symptoms. Participants were asked to record any cold and flu symptoms each day of the week using a set of codes provided with the log. This method was chosen to eliminate participant bias when recording from memory. The parents and/or participants were asked to return the health log once completed. The total number of days per week with URTI symptoms was then calculated for each subject, with days being counted only if two or more consecutive days of cold or flu symptoms were reported (Corbett et al. 2010; Nieman et al. 1998).

3.3.2 Saliva Analysis

One milliliter of unstimulated whole mixed saliva was collected from each participant every morning using salivette swabs (SARSTEDT Inc., Quebec, Canada). Subjects were instructed to moisten/chew lightly on the swab for one minute. After sampling, the swabs were placed directly into plastic tubes. Once all samples of each week were collected, the tubes were centrifuged at 3000xg for 10 minutes where impurities were filtered out and the resulted saliva sample was alliquotted into two separate 1.5 ml eppendorf tubes and stored at -80°C until analysis.

Mucosal sIgA was assayed in duplicate by commercially available ELISA kits (Salimetrics, LLC, Pennsylvania, U.S.A). Due to recent evidence that concentrations of sIgA, as measured by ELISA, may be affected by the use of salivettes (Strazdins et al. 2005) a normal set of standards was compared with a second set of standards (10 µl of standard in 4mL of 1X sIgA diluent) that was run through a set of six salivettes, and

centrifuged for 10 minutes at 3500 rpm to separate the standards from the swabs. The two sets of standards were then compared, and found to be no different.

Cortisol and testosterone were assayed using an in-house assay in the Department of Psychology at Brock University using methods described elsewhere (Carré et al., 1996). Cortisol (R4866) and testosterone (R156/7) antibodies and corresponding horseradish peroxidase conjugates were obtained from C. Munro Clinical Endocrinology Laboratory (University of California Davis, USA). Steroid standards were obtained from Steraloids Inc. (Newport, Rhode Island, USA). The intra-assay and inter-assay coefficients of variation for each of the assays were: 7.1% and 6.3% for sIgA, 6.5% and 6.8% for testosterone, and 7.8% and 6.5% for cortisol, respectively.

3.4 Statistical Analysis

The variability of the stress hormones was measured by their percent coefficient of variation (%CV). A repeated measures ANOVA (week x day) was used in order to examine changes in sC, sT, T/C ratio, and sIgA. To control for missing data, a listwise deletion was applied so that subjects with missing daily values were completely ignored when running the repeated measures model. Therefore, the final number of subjects that had valid values for all variables and used in this analysis was 21. Following this, the hormonal and immune changes for the subset of swimmers who did not report URTI symptoms at the beginning of the study (first week) were further examined using the same steps (n=18).

Paired t-tests were used to compare the mean weekly responses of each marker.

Pearson's product moment correlations were used to determine potential associations between markers of biological stress and mucosal immunity. An alpha level of ≤ 0.05 was used as the criterion for significance for all statistical analyses, which were conducted using SPSS version 19 for Windows (SPSS Inc., USA).

Chapter 4: Results

Participants' physical characteristics such as age, body mass, height, and percent body fat (%BF) are presented in Table 1. There was a significant difference in %BF between boys and girls. The classification of the participants based on sexual maturation (Tanner stage) is presented in Table 2. Sixty percent (60%) of the boys were classified as late pubertal (Tanner stage 4) while the girls were less mature as a group; 38% early pubertal (Tanner stage 2), 31% pubertal (Tanner stage 3) and 31% late pubertal. Despite this difference in maturity, when age, Tanner stage and gender were entered in the repeated measures model as covariates, no significant effect was observed so the data were pooled together for the final analysis.

Table 1. Participants' physical characteristics (n=23)

Variables	Boys n=10	Girls n=13	Total Cohort n=23
Age	13.9± .3	13.4± .3	13.6± .2
Body Mass (kg)	56.4±3.6	54.9±3.0	55.4±2.2
Body Height (cm)	168.4±3.7	164.2±2.2	166.1±2.0
Percent Body Fat (%BF)	11.3± .8	18.8±1.3*	15.6±1.1

**significant difference in %BF between boys and girls, P≤ 0.05.*

Table 2. Sexual Maturity by Tanner stage (number of subjects)

Stage	Boys	Girls[1]	Total
1	1	0	1
2	1	5	6
3	2	4	6
4	6	4	10

[1]**Post-menarcheal** *n =7*

Weekly mean values of hormonal and immune biomarkers are presented in Table 3. Salivary Testosterone (sT) was significantly lower during the experimental week while the 13% increase of sIgA was not statistically significant. The percent coefficient of variation (%CV) of all the hormonal and the immune markers are presented in Table 4. The variability of hormones was no different between weeks. Significantly lower %CV was found for sIgA during the experimental week.

Table 3. Hormonal and immune markers including salivary Cortisol (sC), salivary Testosterone (sT), Testosterone/Cortisol ratio (T/C), and salivary immunoglobulin A (sIgA). Data are presented in mean weekly values (SE).

Variables	Control Week (N =23)	Experimental Week (N =23)
sC (ng/ml)	2.70 ± .22	2.52 ± .23
sT (pg/ml)	181.32 ± 11.49	154.54 ± 11.32*
T/C (ratio)	83.38 ± 7.04	77.88 ± 7.69
sIgA (ug/ml)	47.87 ± 4.42	54.88 ± 5.21

**significant difference (P≤ 0.05).*

Table 4. Percent Coefficient of variation (%CV) of salivary Cortisol (sC), salivary Testosterone (sT), Testosterone/Cortisol ratio (T/C), and salivary immunoglobulin A (sIgA).

Variables	Control Week	Experimental week
sC (%)	36.92 ± 2.4	37.65 ± 2.96
sT (%)	32.75 ± 4.09	26.37 ± 3.4
sIgA (%)	46.47 ± 4.23	35.85 ± 5.02*

**significant difference (P≤0.05).*

Figures 1, 2, 3, and 4 present the daily values of sC, sIgA, sT, and T/C ratio, respectively during the control and the experimental week. Repeated measures, two-way (week x day) ANOVA showed no significant effect of week or day for sC, T/C and sIgA. Testosterone showed a significant ($p<0.05$) week effect with significantly lower levels during the competition week for days 2, 4, 5, 6 and 7. There was no significant week x day interaction for any of the biomarkers.

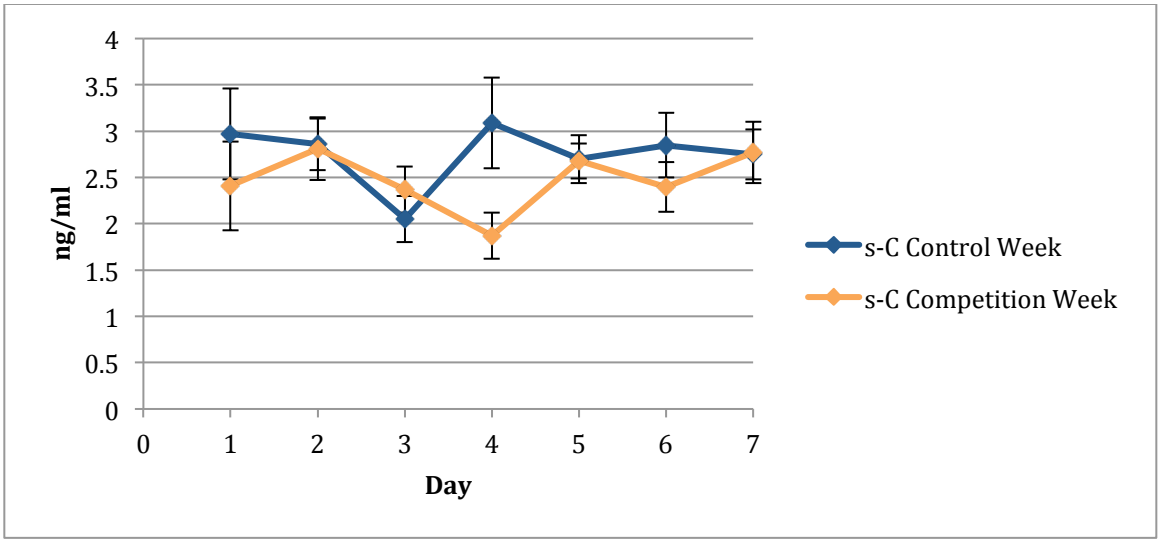

Figure 1. Salivary Cortisol (sC) levels during the control and competition week.

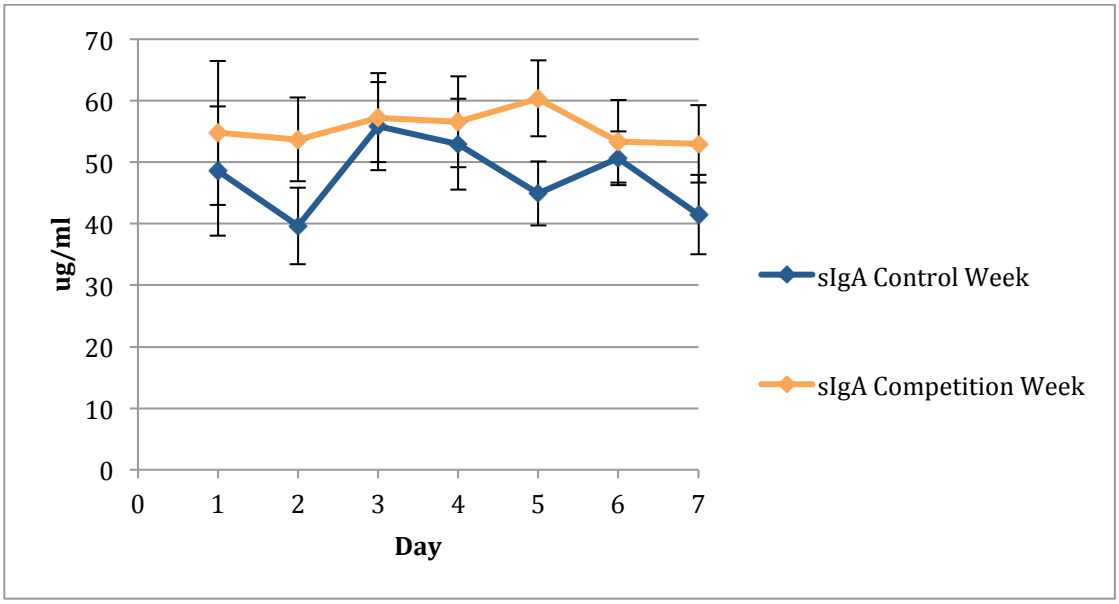

Figure 2. Salivary Immunoglobulin (sIgA) levels during the control and competition week.

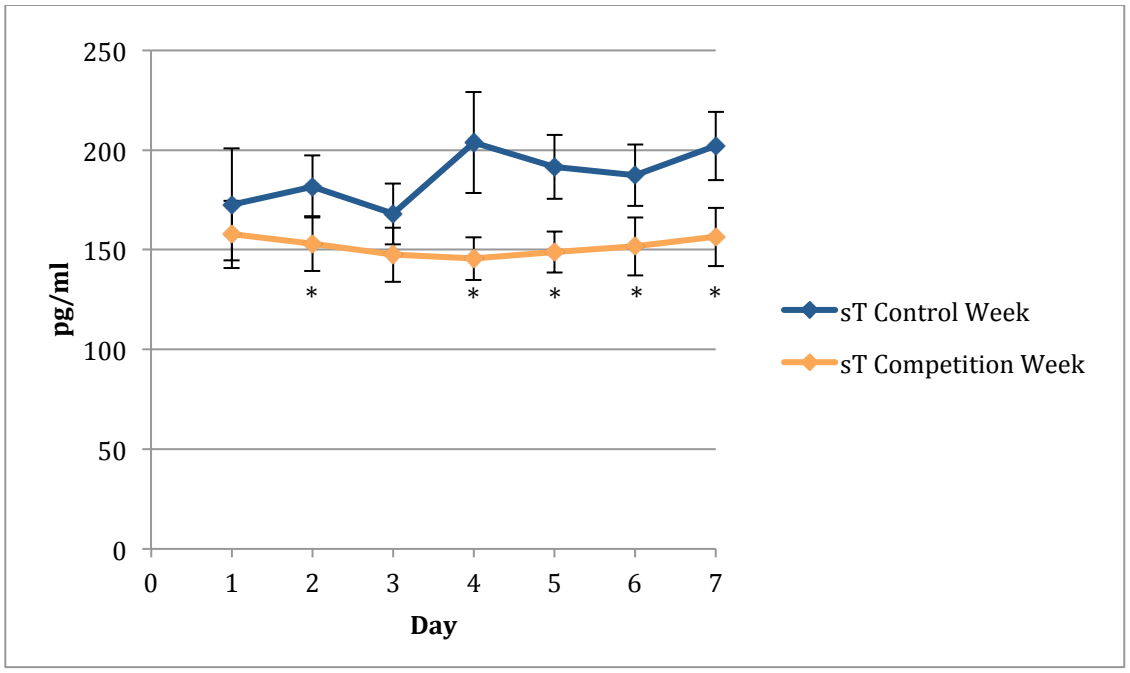

Figure 3. Salivary Testosterone (sT) levels during the control and competition week.

*Significant differences between control and experimental week (P<0.05).

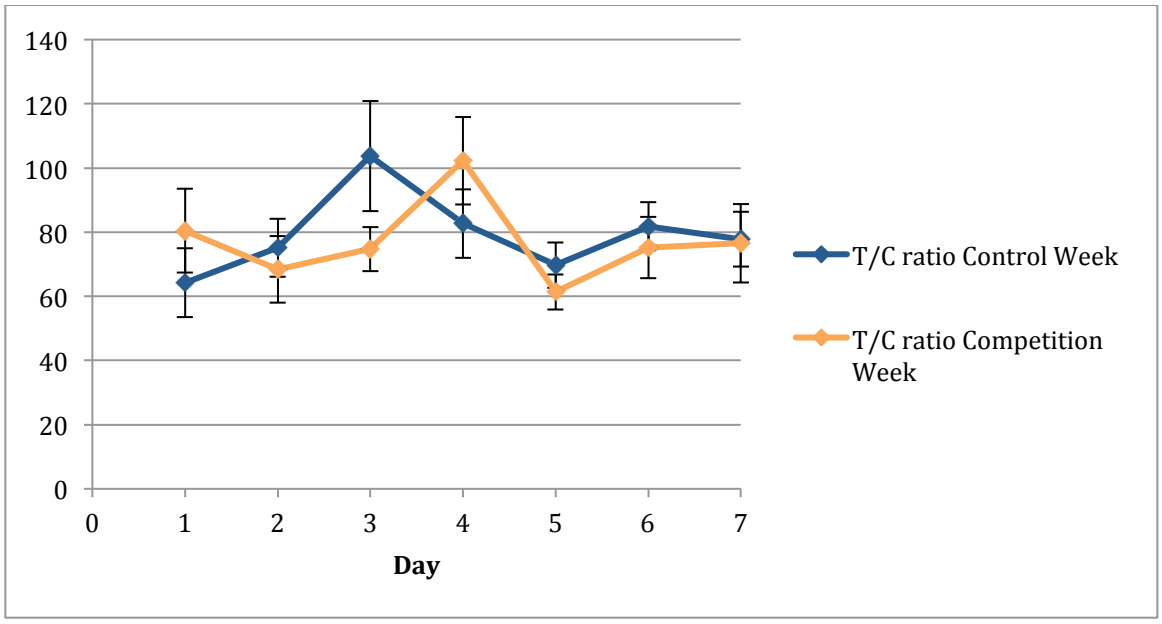

Figure 4. T/C ratio during the control and competition week.

Table 5 presents the results of the anxiety scale. There were no significant differences in the scores between weeks. In addition, all swimmers improved their performance times. Specifically, taking into account all events performed during the two days of competition, the mean percent improvement between seeding times and final times was 6.2% (SD 3.1).

Table 5. Results of the Sport Anxiety Scale 2 during both weeks. Data are mean values (SE).

	Control Week	**Experimental Week**
Total Score	26.8 ± 1.89	27.85 ±1.8
Somatic Anxiety	9.05 ± .8	9.35 ± .74
Worry	10.4 ± .9	11.2 ± .98
Concentration Disruption	7.2 ± .55	7.05 ± .51

Five of the twenty-three swimmers reported URTIs during the control week and the mean number of days with URTI symptoms per subject was 3.0±1.0. Similarly, five other swimmers also reported URTIs during the week of competition and mean number of days with URTI symptoms per subject was 3.8±1.0. Pearson correlation analysis indicated that during the control week the number of days with URTI symptoms was positively correlated with the weekly mean sC ($r=0.45$; $p<0.05$) and sIgA ($r=0.46$; $p<0.01$) and negatively correlated with T/C ratio ($r=0.49$; $p<0.05$). During the competition week, number of days with URTI symptoms was positively correlated with sC ($r=0.50$; $p<0.05$). Moreover, when URTI incidence was entered as a covariate in the

repeated measures a significant effect was observed. Because of this, we run a separate analysis for those subjects who did not report URTI symptoms at the beginning of the study (first week) (n=18). As evidenced in Table 6 the results of swimmers without URTI symptoms the results were consistent with what was found in the total cohort of swimmers.

Table 6. Hormonal and immune characteristics in Healthy Subjects during the control and experimental week.

	Control Week	Experimental Week
Variables	**Healthy Swimmers (n=18)**	**Healthy Swimmers (n=18)**
sC(ng/ml)	2.46 ± 0.21	2.56 ± 0.29
sT(pg/ml)	180.12 ± 15.93	150.56 ± 13.94*
T/C ratio	81.03 ± 8.99	70.40 ± 8.24
sIgA (ug/ml)	44.09 ± 4.54	54.86 ± 5.86

**significant difference (P≤0.05).*

Chapter 5. Discussion

The aim of this study was to determine if a relationship exists between stress and immunity in elite young swimmers during a week leading to a major competition. To our knowledge, there is no study that examined the chronic hormonal and immune responses in young athletes prior to a significant competition. Opposite to our expectation, there was no difference in salivary cortisol levels and no change in its variability between a typical, non-competitive week and a week leading to a significant competition in this group of young swimmers. It is possible that the reduced amount of training (tapering) started late or was not enough to induce a decrease in cortisol levels. Alternatively, the tapering effect on cortisol counteracted the anticipated competition-induced increase in cortisol and explains why cortisol variability also did not increase. Similarly, there were no significant differences in sIgA and incidence of URTI between weeks and so no relationship was found between stress and immunity in these young swimmers. However, testosterone levels were lower during the competition week compared to the control week, along with a 7%, non-significant decrease in the weekly T/C. This may reflect some residual fatigue from previously intense training or insufficient/late tapering that might have affected the anabolic response of the swimmers while preparing for competition. This hormonal state, on the other hand, did not have a negative effect on the swimmers' performance.

5.1 Sexual Maturation

Sixty percent (60%) of the boys were classified as late pubertal (Tanner stage 4) while the girls were less mature as a group. This may be associated with the selection bias previously reported in high performance youth sports (Bass et al. 2000; Baxter-Jones and Helms 1994). Coaches prefer to choose early mature boys due to their favorable muscle mass development that positively contribute to their performance (Baker and Logan 2007; Carling et al. 2009; Gurd and Klentrou 2003; Moore et al. 2010). In contrast, later maturing girls seem to be selected as more likely to be successful in some sports (Baxter-Jones and Helms 1996; Klentrou 2006; Malina et al. 1997). Despite the maturity differences, when age, maturation stage and gender were entered in the repeated measures model as covariates, no significant effect was observed.

5.2 Stress response

The swimmers' cortisol levels corresponded to the typical sC values for this specific age range (11-15 years old) with no differences between boys and girls (Chatard et al. 2002; Price et al. 1983; Tornhage et al. 2002). It was hypothesized that there would be an increased variability in stress hormones with sC gradually increasing and the T/C ratio decreasing the week of competition. However, the results showed that neither the absolute salivary cortisol levels nor the %CV of sC significantly differed between the control and the competition week. There are two possible explanations for the lack of difference in cortisol levels between the two weeks; either the control week did not

reflect true baseline levels, or this major competition was insufficient to induce a significant stress response to the young athletes. This is in agreement with Lac et al. (2000), who also did not find significant changes in cortisol levels prior to an endurance competition in adult athletes. Furthermore, in our study cortisol was not significant different during the days of competition, which is in contrast to the majority of the literature that have reported an increase of cortisol the days of significant competitions in comparison to typical resting days (Aubets et al 1995, Filaire et al. 2001, Moreira et al. 2013, Salvador et al. 2003). Nevertheless, it is possible that the reduced training volume during the competition week masked the competition effect on salivary cortisol keeping it at the same level as when the training volume was higher during to the control week. Furthermore, there was a small but not significant difference on the fourth day between the two weeks. Specifically, day 4 was characterized by higher sC levels during the control week as compared to the week of competition. This may be attributed to the difference in training volume between the two weeks. Cortisol levels might have increased during the control week due to higher training volume while the decreased sC the week of competition might be the result of tapering by this specific day.

In the absence of stress, however, and given that training volume was lower by 25%, one would expect cortisol to decrease during the competition week as previously suggested (Filaire et al. 2003). In their study, Filaire et al. (2003) found that cortisol decreased in response to recovery periods (tapering) in comparison to periods of intense training in soccer players of various ages. They suggested that a fast HPA adaptive response might occur in athletes in response to a tapering phase irrespective of an athletes' chronological age. Other studies investigating the cortisol responses of young

athletes to training and/or competition (Filaire et al. 2009, Georgopoulos et al. 2011,, Mortatti et al.2009) have reported contradictory results. Mortatti et al. (2009) investigated the impact of a competition and training on hormonal and immune parameters in young soccer players over a period of seven games in 20 days. They found no significant changes in sC levels, despite increments in training load. Similarly, when comparing sC levels before and after 16 weeks of training and competition in young female tennis players, Filaire et al. (2013) found that there was a reduction in this parameter at awakening and 30 minutes after awakening, which could be related to chronic stress. Prolonged, chronic stress has been found to be associated with a blunting of the cortisol level at this time of day (Huber et al. 2006), which is the same time we measured sC in young swimmers.

Salivary testosterone significantly decreased the week of competition in comparison to the control week, indicating that the majority of the swimmers had a compromised anabolic response. Testosterone's behavior prior to a competition is not well established in the literature. Filaire et al. (2001) found that salivary testosterone did not change before a competition in judoka. Others, however, reported elevated levels of testosterone in adult tennis players before a match (Booth et al. 1989). According to Maso et al. (2004), testosterone is influenced by tiredness with low testosterone levels being an indication of fatigue. Thus, the lower testosterone levels during the experimental week may reflect that the young swimmers were tired maybe due to a residual effect of the intense training prior to the week of competition meaning insufficient or late tapering the days prior to competition. In a recent study, Papacosta et al. (2013) examined the time-course of change of sT, sC and sIgA, mood state and performance during a 2-week

taper in judo athletes, and found that changes in hormonal responses, precede enhancements in performance and mucosal immunity, suggesting that judo athletes should taper for at least a week prior to competition.

In accordance with the lower testosterone levels, T/C ratio also decreased by 7% during the competition week. According to Filaire et al. (2001), this ratio is considered to reflect states of anabolism when it is high, and states of catabolism when it drops by 30% or more. Evidence of another study shows that a decreased T/C ratio prior to competition does not automatically lead to a decrease in team's performance (Filaire et al. 2001) even though a decreased T/C ratio is an indication of overtraining (Budgett 1998). So given the slight decrease of the ratio the week of competition it is not surprising that all swimmers improved their times.

The swimmers did not score high on the sport anxiety scale and their scores match the mean values for their age (Smith et al. 2006). In addition, all swimmers improved their times despite the decreased testosterone the week of competition.

5.3 Mucosal Immunity

It was hypothesized that sIgA counts will be decreasing getting closer to competition. The results did not support our hypothesis. In fact, during the competition week, sIgA slightly increased and its variability as determined by the %CV was significantly lower.

Strong evidence indicates that prolonged intense exercise results in suppressed sIgA levels and this is associated with increased risk of URTI incidence (Gleeson et al. 1995, Tharp et al. 1990, Tsai et al. 2011). Our results, however, showed that during the

control week, swimmers had normal sIgA according to the physiological typical values within this specific age range (Fadel et al. 2013, Rashkova et al. 2010). On the other hand, sIgA was significantly correlated with URTI symptoms leading to the suggestion that the slight increase in sIgA during the week of competition (13% in the total cohort and 18% for the those who were healthy during the control week) was an acute response to the increased total number of days with URTI symptoms during the same week, but it doesn't seem to be the result of chronic immunosuppression. Nevertheless, the number of swimmers reporting symptoms of URTI was the same during both weeks. Nakamura et al. (2006) also reported no significant relationship between sIgA and appearance of URTI. Another study, conducted by Novas et al. (2003) supports the results of our study that elite young athletes may have higher risk of URTI symptoms during periods of important competition. Swimmers had followed prolonged intense exercise regimen before the week that was leading to the significant swim meet so the increased total number of days with URTI symptoms may be associated with factors such as fatigue or not enough tapering before the competition. Nevertheless, no immunosuppressive effect was observed in this population due to competition.

5.4 Interaction between markers of biological stress and mucosal immunity

It was hypothesized that competition will increase biological stress and this would have a negative effect on mucosal immunity in these young swimmers. Specifically, stress levels and the variability of stress hormones would increase closer to competition and this would have a negative effect on mucosal immunity. Although salivary cortisol was

positively correlated with URTI we found no relationship between stress and sIgA in this age group. Therefore, this correlation may indicate that the body is simply under stress when producing antibodies to fight a pathogen. Numerous studies tried to examine this relationship although there is no unanimity pertaining to this association. Cieslak et al. (2003) suggested that stress does not have a negative effect on mucosal immunity in children while physical activity does. Tiollier et al. (2005), who also found no significant correlation between salivary cortisol and sIgA, have suggested that cortisol levels do not differ between healthy and ill adults. He et al. (2010) found that during a basketball season immunity was suppressed and cortisol was significantly increased. A recent study by Moreira et al. (2013) reported that sIgA was significantly lower while cortisol increased before a significant match in volleyball players. An inverse relationship between cortisol and sIgA was also found by Hucklebridge et al. (1998). However, the effect of stress on the immune system in younger athletes is less evident.

5.5 Strengths and Limitations

The present study has two major strengths. The swimmers who participated in the study were similar in terms of both competitive level and training volume, which resulted in a very homogeneous study group. The second strength was the consistent season and the time of sampling. All samples were collected within a short time frame between the end of November and mid-January to avoid seasonal variations. In addition, the swimmers provided all the saliva samples right after awakening and before breakfast. Thus, these resting morning saliva samples were not affected by the diurnal rhythm and neither the

immune or hormonal components were influenced by hydration or food. On the other hand, Huber et al. (2006) found that prolonged, chronic stress was associated with a blunting of the cortisol level at this time of day in depression patients. Another strength of the study was the sample size, which resulted to high statistical power (>0.80) for sT. T/C and sIgA. The only exception was sC for which a sample size of 44 would be needed to achieve a power equal to 0.80.

One of the limitations of the present study includes the inconsistency in the self-collection of saliva, and the compliance of the swimmers to the daily task of collection. Although competition induced anxiety was a secondary, control measure, the Sport Anxiety Scale-2 questionnaire might not have been a good choice of anxiety assessment before the competition for two reasons: a) it reflects trait anxiety, as opposed to state anxiety, and b) the questions are not relevant to the sport of swimming. The questions refer to "game", and "play", rather than "competition" and "swim". A more relevant tool to document competition related anxiety in the swimmers would be more appropriate. Likewise, as a limitation is considered the fact that we did not assess perceived stress or stress related to other life events.

5.6 Conclusion

Our findings indicate that in the absence of stress mucosal immunity was unaffected in this group of young swimmers during a week leading to competition. The normal anxiety scores in conjunction with the unchanged cortisol levels and variability of both cortisol and T/C ratio during the study suggest that swimmers were not significantly stressed by

the swim meet. Based on their training and competition background, these high performance swimmers were familiar with the conditions of a significant swim meet. However, the fact that cortisol did not decrease during the competition week despite the lower amount of training may indicate that swimmers did experience some kind of stressing situation during the swim meet.

In the week of competition, salivary testosterone decreased. The lower testosterone is attributed to the fact that swimmers had experienced a protracted intense training the weeks before the competition with insufficient or late tapering and, therefore, their testosterone levels decreased as a subsequent phenomenon of fatigue. However, this small shift in their apparent metabolic state did not negatively influence the swimmers' performance in the competition.

5.7 Significance of the Study and Take Home Message

The relationship between stress and immunity in athletes is an area of interest, which is clouded by conflicting results. The present study tried to provide answers pertaining to this association. The findings of the present study reflect how stress and immunity respond during a week leading to and including competition in elite young swimmers. Competition stress seems to be low in top level, peri-pubertal athletes and immune factors such as sIgA do not seem to be influenced by competition. Elite young athletes were able to cope with the decreased testosterone without having issues in their performance at the competition.

5.8 Future recommendations

Further research is needed to examine the effects of chronic stress on salivary markers of immunity in young athletes. Lastly, a future study is needed to examine the relationship between stress and immunity in adult versus children and adolescent athletes leading to competition. This way we would be able to better understand the difference in the stress response between young and adult athletes.

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APPENDICES

APPENDIX A

Invitation Letter

INTERACTION BETWEEN STRESS AND IMMUNITY IN YOUNG SWIMMERS

Principal Investigators: Dr. Nota Klentrou and Makis Papadopulos, Department of Kinesiology, Brock University.

We would like to invite you to participate in the present study, which investigates the interaction between stress and immunity in young swimmers.

The **purpose** of this research project is to examine the interaction between stress and immunity in young swimmers during a week leading to competition and compare it to a non-competitive week.

The study will require daily saliva samples from your child during two separate weeks between January and February. These samples will be self-collected using specifically designed swabs which will be brushed up against your child's cheek. Your child will be asked to provide a saliva sample every morning after awakening and before breakfast during a typical, non-competitive week and during a week leading to a significant swim meet. Additional measurements will include the self-assessment of pubertal maturity and filling out other questionnaires.

Participation in this project will allow the opportunity to experience research and contribute to the advancement of science.

If you have any pertinent questions about your rights as a research participant, please contact the Brock University Research Ethics Officer (905 688-5550 ext 3035, reb@brocku.ca)

If you have any questions, please feel free to contact us.

Thank you

Principal Investigators:

Dr. Nota Klentrou and Makis Papadopoulos
Department of Kinesiology
Faculty of Applied Health Sciences
Brock University
Tel: 905-688-5550 ext: 4538 or 5623
email: nklentrou@brocku.ca or mp11ub@brocku.ca
This study has been reviewed and received ethics clearance through Brock University's Research Ethics Board (file 05-155)

APPENDIX B

STUDY INFORMATION AND CONSENT/ASSENT

INTERACTION BETWEEN STRESS AND IMMUNITY IN YOUNG SWIMMERS

You and your child swimmer are being invited to participate in a research study being conducted by the investigators listed below. Prior to participating in this study, please read this form to find out about the purpose and the tests of this study. This study is sponsored by the Faculty of Applied Health Sciences of Brock University.

INVESTIGATORS:	**DEPARTMENT:**	**CONTACT:**
Dr. Nota Klentrou	Kinesiology	(905) 688-5550 x4538
Makis Papadopoulos	Kinesiology	(416) 571 9935

PURPOSE:

The overall purpose of this study is to examine the interaction between stress and immunity in young swimmers. The specific objectives are to examine: a) the variability of sIgA, salivary immunoglobulin M (sIgM), salivary lactoferrin (sL), salivary cortisol (sC) and salivary testosterone (sT) in young swimmers during a week leading to competition and compare it to a control, non-competitive week, and b) the effect of competition on mucosal immunity in young athletes.

DESCRIPTION OF PROCEDURES:

All swimmers enrolled in the study will be asked to complete the following measurements:

1. Questionnaires: The swimmer will be asked to complete questionnaires outlining their medical history and pubertal status. The questionnaire used to measure pubertal status involves your child looking at drawings of male or female genitalia and deciding which stage of puberty they best match. This will be carried out in a private room to avoid any uneasiness. Also, please be aware that the medical history questionnaire includes questions about medications, alcohol use, and smoking. The day of competition the swimmers will be asked to fill the Sport Anxiety Scale-2 for children. In all questionnaires, your child may choose not to answer a question without penalty.
2. Body Composition: Your child's height, weight, and body fat percentage will be measured. Percent body fat will be estimated using skinfold thickness (mm) assessed at two sites, the arm and upper back (triceps and subscapular).

3. Saliva: One saliva sample will be collected every morning from your child to determine the effects of stress and to determine secretory immunity. This sample will be collected specifically designed swab which will be brushed up against your child's cheek. Your child will be asked to provide a saliva sample every morning after awakening and
   before breakfast during a typical, non-competitive week and during a week leading to a
   significant competition. The variability and the ratio of resting levels of salivary hormones Cortisol and Testosterone will be measured to determine the effects of stress. Secretory immunity will be assessed by resting levels of salivary immunoglobulins A and M, which are antibodies, as well as another immune factor namely lactoferrin.
4. Swimming times will be recorded at the competition to examine changes in performance.

CONFIDENTIALITY:

All data collected during this study will remain confidential and will be stored in offices and on secured computers to which only the

principal and co-investigators have access. You should be aware that the results of this study will be made available to scientists, through publication in a scientific journal, but your name and any personal data will not appear in compiling or publishing these results. Data will be kept for 5 years after the date of publication, at which time all information will be destroyed.

PARTICIPATION AND WITHDRAWAL:

You and your child can choose whether to participate in this study or not and may remove your data from the study if you wish. Withdrawing from the study will not affect your or your child's status in any other program offered by the University. Your child may also refuse to answer any questions posed to them during the study and still remain as a participant in the study.

RISKS AND BENEFITS

The only foreseeable risks involved in participation include:

a) Possible skin irritation from cleaning the skin with alcohol and applying surface electrodes. This can be minimized by washing the skin and applying skin lotion.
b) Some questionnaires may pose a potential embarrassment. In such a case, your child need not reply to any question they do not wish to.

Participation will allow your child to become exposed to a research protocol, contribute to the advancement of science. Additionally, if an unusually low or high result is attained for any of the measurements, reflecting a possible health-related problem, you will be alerted and advised to consult your physician.

RIGHTS OF RESEARCH PARTICIPANTS:

You will receive a signed copy of this consent form. You may withdraw your consent to participate in this study at any time, and you may also discontinue participation at any time without penalty. In signing this consent form or in participating in this study you are not waiving any legal claims or remedies. This study has been reviewed and received clearance from the Brock University Research Ethics Board (file #05-155). If you have any pertinent questions about your rights as a research participant, please contact the Brock University Research Ethics Officer (905 688-5550 ext 3035, reb@brocku.ca)

INFORMATION:

Please contact Dr. Nota Klentrou at 905-688-5550(X4538), or Makis Papadopoulos at 416-571-9935 if you have any questions about the study.

CONSENT/ASSENT:

I HAVE READ AND UNDERSTAND THE ABOVE EXPLANATION OF THE PURPOSE AND PROCEDURES OF THE PROJECT. I HAVE ALSO RECEIVED A SIGNED COPY OF THE INFORMATION AND CONSENT FORM. MY QUESTIONS HAVE BEEN ANSWERED TO MY SATISFACTION AND I AGREE TO PARTICIPATE IN THIS STUDY.

PRINTED NAME OF PARTICIPANT	DATE
SIGNATURE of PARTICIPANT	DATE
SIGNATURE of PARENT/GUARDIAN	DATE
WITNESS	DATE

PRINTED NAME OF WITNESS

INVESTIGATOR

In my judgment the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent and participate in this research study.

SIGNATURE OF INVESTIGATOR DATE

APPENDIX C

SUBJECT SCREENING AND MEDICAL HISTORY QUESTIONNAIRE

Name: _____ Date: _____

Date of Birth: _____

Your responses to this questionnaire are confidential. If you answer "YES" to any of the following questions, please give additional details in the space provided and discuss the matter with one of the investigators. You may refuse to answer any of the following questions.

1. Have you ever had any major joint instability or ongoing chronic pain such as in the knee, back or elbow?	**YES**	**NO**
2. Are you currently taking any medication (including aspirin) or have you taken any medication in the last two days?	**YES**	**NO**
3. Have you taken any medication in the past six months?	**YES**	**NO**
4. Is there any medical condition with which you have been diagnosed and are under the care of a physician (e.g. asthma, diabetes, anorexia)?	**YES**	**NO**
5. Do you, or have you in the past, consumed any alcohol on a regular basis?	**YES**	**NO**
6. Do you, or have you in the past, smoked on a regular basis?	**YES**	**NO**
7. Are you, or have you in the past, engaged in any extreme diet?	**YES**	**NO**
8. Do you, or have you in the past, consumed any nutritional supplements (e.g. calcium, multi-vitamin) on a regular basis?	**YES**	**NO**
9. Do you, or have you in the past, engaged in physical activity on a regular basis?	**YES**	**NO**
10. Have you had any fractures?	**YES**	**NO**

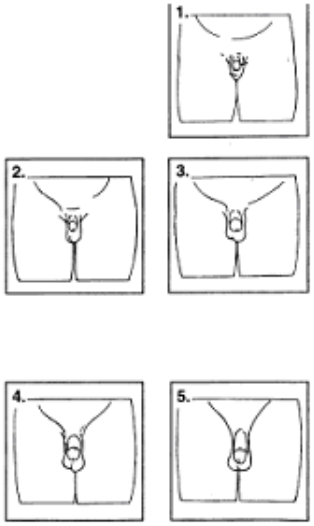
APPENDIX D

SEXUAL MATURATION AUTOEVALUATION QUESTIONNAIRE (BOYS)

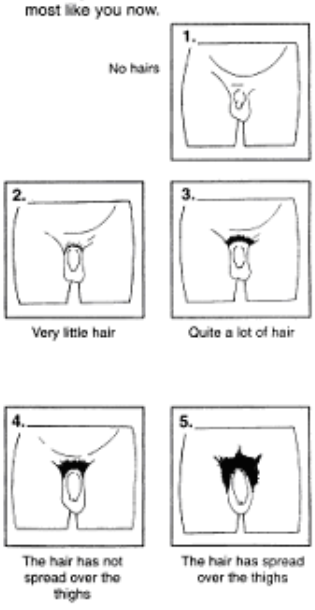
Directions: You should choose only one of the stages shown below. One stage for genital development and one stage for pubic hair development.

Study Subject No:

please put a tick in the box that looks most like you now



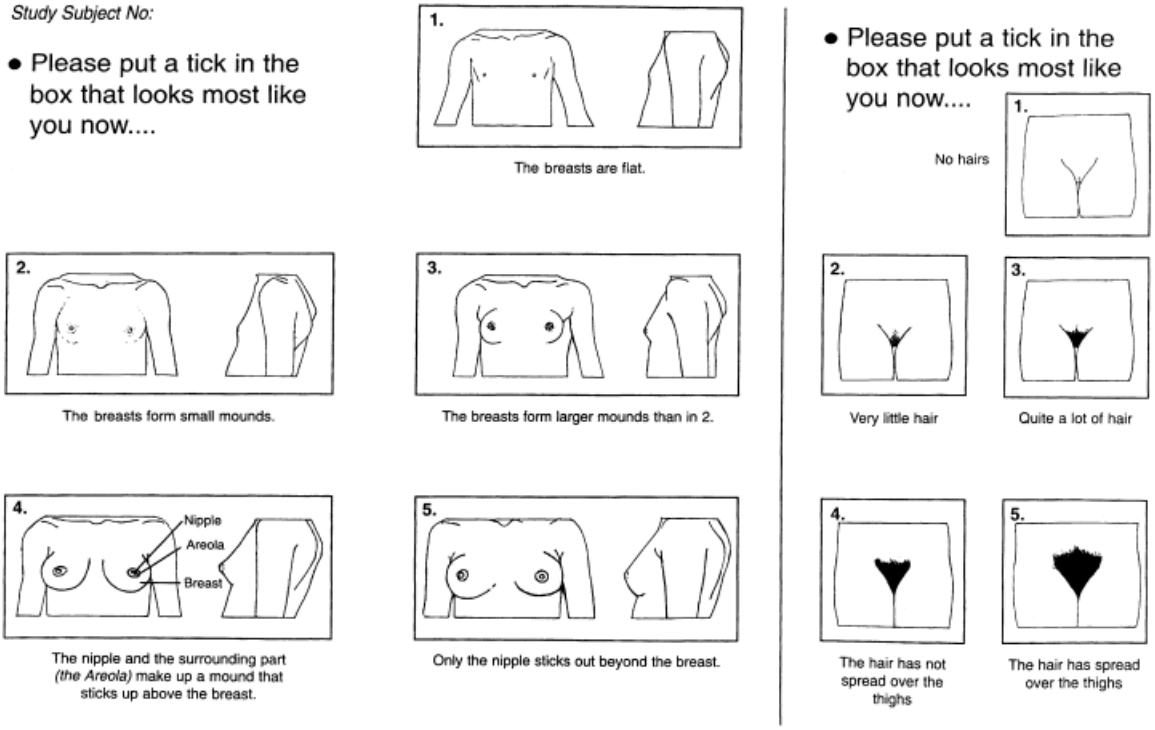
- Please look at the **Pubic Hair** only in these pictures.
- Please put a tick in the box that looks most like you now.



From Taylor et al, 2001.

SEXUAL MATURATION AUTOEVALUATION QUESTIONNAIRE (GIRLS)

Directions: You should choose only one of the stages shown below. One stage for Breast development and one stage for Pubic Hair development.



From Taylor et al, 2001.

Please answer the following questions:

1. Have you had your period?

   YES NO

2. If yes, how old were you when you had your first period? _____

3. How often do you get periods? (in days)_____

APPENDIX E

Sport Anxiety Scale-2

REACTIONS TO PLAYING SPORTS

Many athletes get tense or nervous before or during games, meets or matches. This happens even to pro athletes. Please read each question. Then, circle the number that says how you USUALLY feel before or while you compete in sports. There are no right or wrong answers. Please be as truthful as you can.

Before or while I compete in sports:	**Not At All**	**A Little Bit**	**Pretty Much**	**Very Much**
1. It is hard to concentrate on the game.	1	2	3	4
2. My body feels tense.	1	2	3	4
3. I worry that I will not play well.	1	2	3	4
4. It is hard for me to focus on what I am supposed to do.	1	2	3	4
5. I worry that I will let others down.	1	2	3	4
6. I feel tense in my stomach.	1	2	3	4
7. I lose focus on the game.	1	2	3	4
8. I worry that I will not play my best.	1	2	3	4

Before or while I compete in sports:	Not At All	A Little Bit	Pretty Much	Very Much
9. I worry that I will play badly.	1	2	3	4
10. My muscles feel shaky.	1	2	3	4
11. I worry that I will mess up during the game.	1	2	3	4
12. My stomach feels upset.	1	2	3	4
13. I cannot think clearly during the game.	1	2	3	4
14. My muscles feel tight because I am nervous.	1	2	3	4
15. I have a hard time focusing on what my coach tells me to do.	1	2	3	4

Scoring Key. Somatic: Items 2, 6, 10, 12, 14; Worry: Items 3, 5, 8, 9, 11; Concentration Disruption: Items 1, 4, 7, 13, 15.

APPENDIX F

Health/Sickness Log

Name _____

Mailing Address:

City: _____ State: _____ Zip:

Age _____ years; Height _____ feet _____ inches; Body weight _____ pou

1. ☐ **Yes;** ☐ **No** Did you get a *flu shot* during the last 12 months?

2. ☐ **Yes;** ☐ **No** Are you involved with a competitive sport?

3. If YES, which one: _____

4. How many hours per week do you train? _____ hours

Health Log: Month of November 2012

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
				1	**2**	**3**
4	**5**	**6**	**7**	**8**	**9**	**10**
11	**12**	**13**	**14**	**15**	**16**	**17**
18	**19**	**20**	**21**	**22**	**23**	**24**
25	**26**	**27**	**28**	**29**	**30**	

Instructions:

Fill in **health codes(s)** (as many as apply).

1. No health problems today.
2. COLD SYMPTOMS (runny, stuffy nose, sore throat, coughing, sneezing, colored discharge)
3. FLU SYMPTOMS (fever, headache, general aches and pains, fatigue and weakness, chest discomfort, cough)
4. Nausea, vomiting, and/or diarrhea
5. Muscle, joint, or bone problems/injury
6. Other health problems (describe)

Please rate severity of symptoms:

A=mild; B=moderate; C=severe

For example, for a moderate cold, write "2-B" in the blank for the specific days.

Month of December 2012

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
						1
2	**3**	**4**	**5**	**6**	**7**	**8**
9	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**
23	**24**	**25**	**26**	**27**	**28**	**29**

Instructions:

Fill in **health codes(s)** (as many as apply).

1. No health problems today.
2. COLD SYMPTOMS (runny, stuffy nose, sore throat, coughing, sneezing, colored discharge)
3. FLU SYMPTOMS (fever, headache, general aches and pains, fatigue and weakness, chest discomfort, cough)
4. Nausea, vomiting, and/or diarrhea
5. Muscle, joint, or bone problems/injury
6. Other health problems (describe)

Please rate severity of symptoms:

A=mild; B=moderate; C=severe

For example, for a moderate cold, write "2-B" in the blank for the specific day

APPENDIX G

FOLLOW THE INSTRUCTIONS

1. PLACE THE ZIPLOCK BAG LABELED "EMPTY SALIVETTES" BESIDE YOUR BED!
2. PLACE THE ZIPLOCK BAG LABELED "FULL SALIVETTES" IN THE KITCHEN'S **FREEZER** (NOT IN THE FRIDGE)!
3. REPEAT THE STEPS EVERY MORNING **RIGHT AFTER YOU WAKE UP!**
4. CHECK EVERY STEP ONCE YOU COMPLETE IT!
5. DO NOT FORGET TO INITIAL EVERY STEP AND SIGN EVERY DAY!!!

Date:

CHECK	TASK	INITIAL
	Take the correct salivette from the first bag (the day is on label of the salivette)	
	Open the tube's cup! Put the swab in your mouth and chew it for 1 minute (60sec)	
	Place the swab back in the tube (with your teeth) WITHOUT touching it with your hands!!	
	Close the cup and immediately put the tube in the other bag in freezer!!!	

Signature: _____