UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Dinâmica de Marcadores Salivares de Atividade Autônoma e Adrenocortical em Resposta à Competição de Elite

Aluno: Miguel Mauricio Díaz Gómez

Orientador: Prof. Dr. Foued Salmen Espindola

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(Foued Salmen Espindola)

a Foued Salmen Espindola,
pela oportunidade, a confiança e o respeito.

Índice

Apresentação

Os estudos apresentados nesta dissertação foram delineados para examinar alguns dos eventos biológicos subjacentes à exposição a estímulos adversos (estresse). De interesse particular é a resposta do cortisol salivar, alfa-amilase, cromogranina A e proteína total na saliva à competição esportiva profissional. Foi postulado que a magnitude de variação do cortisol ao acordar indicaria mudanças similares em estados de humor e níveis de desempenho durante o dia (capítulo 2) e que a o comportamento de marcadores de atividade simpática dependia dos mecanismos responsáveis pela sua secreção na saliva e não de propriedades particulares a cada analito (capítulo 3). Uma competição em natação de nível nacional foi utilizada como modelo de estresse. Os participantes (atletas profissionais) foram avaliados ao longo dos dois primeiros dias de competição e durante dois dias consecutivos de treinamento para a obtenção de parâmetros de base. Os resultados de ambos os estudos podem ser resumidos em dois achados principais: 1) a resposta do cortisol ao acordar não necessariamente representa um grau similar de variação em estados de humor e níveis de desempenho e 2) existe uma variação proporcional na concentração de diferentes proteínas na saliva em função de uma maior atividade simpática. Os resultados destes trabalhos sugerem ao leitor diferenças na resposta a estímulos adversos em diferentes populações e técnicas simples para a valoração da reatividade simpática na resposta ao estresse.

Capitulo 1

Fundamentação Teórica

Homeostasia e Estresse

Os seres vivos conseguem manter um estado constante nas propriedades físico-químicas do seu meio interno através de múltiplos mecanismos de auto-regulação. Este estado de equilíbrio dinâmico é denominado 'homeostasia' e é um conceito central ao estudo e entendimento do estresse como processo fisiológico. O estresse é definido como o estado de ameaça, real ou percebida, à homeostasia como conseqüência da exposição a estímulos adversos (estressores)[1].Em resposta a tais estímulos, uma serie de processos fisiológicos e comportamentais é rapidamente iniciada com o objetivo de manter ou re-estabelecer o equilíbrio do meio interno. A atenção, por exemplo, melhora e as funções cerebrais são orientadas ao estimulo estressor. As freqüências cardíaca e respiratória aumentam, o sangue é re-dirigido à musculatura ativa e processos catabólicos de obtenção de energia são priorizados. Estas respostas são geralmente transitórias e tem como objetivo maximizar as chances de supervivência do organismo[2].

O grau de intensidade da resposta a estímulos adversos depende, por sua vez, da gravidade, duração e freqüência do estimulo. Não todos os estímulos adversos provenientes do ambiente conseguem provocar a mesma proporção neste tipo de respostas[3]. Da mesma forma, devido a que em essência, todos os processos biológicos em um organismo estão direta ou indiretamente orientados à manutenção da homeostasia, o conceito de estresse é apropriado em situações imprevisíveis e de ausência de controle que provocam alterações fisiológicas e comportamentais[4].

Efetores Neuroendócrinos da Resposta ao Estresse

A resposta ao estresse é iniciada por uma rede de estruturas neuronais que recebem constantemente informação de altos centros no sistema nervoso central (SNC), a periferia e o ambiente[5]. Este sistema provê informação à glândula pituitária e núcleos pontomedulares que, pela sua vez, controlam a

resposta neuroendócrina e autonômica do organismo por meio dos eixos o hipotalâmico –pituitário -adrenal (HPA) e o simpático –adrenomedular (SAM)[5,6]

Neurônios do núcleo para-ventricular do hipotálamo, da medula e do lócus coeruleus (LC) são os coordenadores centrais da resposta a estímulos adversos[7]. Células do LC e outras células noradrenérgicas da medula são conhecidas como o sistema LC/norepinefrina (NE), responsável por controlar a estimulação do sistema nervoso simpático (SNS) induzida por estresse. No cérebro, a NE funciona como um sinal de alarme que provoca estados pronunciados de excitação e vigilância enquanto diminui funções vegetativas como comer e dormir[7].

O mediador químico mais importante da resposta central ao estresse é o hormônio liberador de corticotropina (CRH)[8]. Estímulos adversos são um potente ativador da liberação de CRH pelo hipotálamo e outros locais como o córtex cerebral, o tronco encefálico e a medula[9]. As células cromafins da medula adrenal são inervadas por nervos pré-ganglionares do nervo esplânico. Tais células medulares produzem catecolaminas[10], as quais circulam no plasma ligadas à albumina e induzem a estimulação do SNS. A ativação do SNS provê um mecanismo de resposta rápida que controla a reação do organismo ao estressor[11]. Além de ativar o SNS, a liberação de CRH durante o estresse, associada à liberação de arginina vasopressina (AVP), resulta em maior secreção de hormônio adrenocorticotrópico (ACTH)[7]. A secreção de ACTH pela glândula pituitária anterior estimula o córtex adrenal para secretar grandes quantidades de glicocorticóides (principalmente cortisol, em primatas)[12]. Os glicocorticóides são o produto final da estimulação do eixo HPA e participam do controle da homeostasia do todo o organismo[13]. Após a sua liberação pelo córtex adrenal em resposta ao ACTH, os glicocorticóides exercem retroalimentação negativa sobre a glândula pituitária, os neurônios produtores de CRH no hipotálamo e o sistema LC/NE[13]. A retroalimentação inibitória dos glicocorticóides limita a exposição dos tecidos a estes hormônios, minimizando

assim seus efeitos catabólicos, lipogênicos, anti-reprodutivos e imunossupressores.

Reações de Fuga ou Luta e Busca de Apoio

A ativação do sistema nervoso durante situações adversas é denominada como a reação de fuga ou luta. A secreção de epinefrina como resultado de uma maior atividade do SNS em situações de perigo e onde é requerida atividade física resulta numa complexa integração de sistemas orientados como mencionado anteriormente, a maximizar as chances de supervivência do organismo. De forma geral, os efeitos da ativação do SNS em resposta ao estresse são[14]:

- Aumento na glicogenólise hepática e muscular.
- Aumento na disponibilidade de gordura como substrato energético.
- Aumento na freqüência e contratilidade cardíaca com subseqüente aumento no debito cardíaco.
- Redistribuição do fluxo sanguíneo desde a musculatura lisa para o músculo esquelético.
- Aumento na freqüência respiratória.
- Aumento dos níveis de fatores de coagulação no sangue.
- Diminuição na motilidade gástrica.
- Melhoria nos níveis de atenção e aumentos nos níveis de ansiedade.

A reação de fuga ou luta, no entanto, é mais característica de machos do que fêmeas. Devido ao papel reprodutivo e de cuidado desenvolvido pelas fêmeas na maioria das espécies, incluindo a humana, a resposta ao estresse em fêmeas está mais relacionada à 'busca de apoio' (tend-and-befriend) do que ao confronto físico ou a fuga em si[15]. Tem sido sugerido que em fêmeas existe de

fato uma ativação do eixo HPA e do SNS em resposta ao estresse. Porém, a mesma é modulada por ocitocina e opióides endógenos permitindo assim às fêmeas sobreviver e cuidar das suas crias sem necessariamente lutar por elas ou abandoná-las[15]. A ocitocina é produzida nos núcleos supra-óptico e paraventricular do hipotálamo e transportada à glândula pituitária onde é liberada na circulação. Pequenas quantidades de ocitocina podem também ser sintetizadas no córtex adrenal e no timo[16]. Por outro lado, ß-endorfinas são produzidas no lobo intermediário da pituitária, no córtex adrenal e em células imunológicas[17,18]. Adicionalmente, as ß-endorfinas regulam a liberação de ocitocina, vasopressina, NE e cortisol, o que possivelmente contribui às diferencias na resposta ao estresse entre machos e fêmeas[15].

Outros hormônios como o glucagon, hormônio de crescimento e aldosterona são também liberados durante a resposta ao estresse enquanto que hormônios com funções anabólicas como a testosterona e a insulina geralmente diminuem[5]. É provável que a taxa de secreção de praticamente todos os hormônios seja alterada durante situações adversas. No entanto, devido a que o cortisol e as catecolaminas são os principais reguladores das respostas de fuga ou luta ou 'busca de apoio', são estes os que com preferência são incluídos no estudo e entendimento da resposta fisiológica e comportamental ao estresse.

Marcadores Salivares

A saliva é um fluido oral complexo produzido e secretado pelas glândulas salivares. Consiste de água (99%), eletrólitos, proteínas e outros metabólitos de baixo peso molecular[19]. A saliva é produzida por três principais pares de glândulas (a parótida, a submandibular e a sublingual) e centos de glândulas menores espalhadas pela cavidade oral. Em essência, a estimulação simpática das glândulas resulta em secreção de proteínas enquanto que a inervação parassimpática é responsável pela secreção de água e eletrólitos[20]. A saliva é fundamental para a preservação e manutenção da saúde dos tecidos orais e tem

diversas funções que variam desde a iniciação da digestão de alimentos até a lubrificação[21].

A saliva apresenta várias vantagens sobre outros biofluidos como o sangue e a urina devido à facilidade de sua coleta e manipulação. Além das substâncias naturalmente presentes na saliva como enzimas digestivas e proteínas antimicrobianas, outras substâncias circulantes no sangue podem se difundir na saliva e ser encontradas em menores concentrações. De particular interesse nesta dissertação é o cortisol salivar. Por ser um hormônio esteróide, o cortisol consegue se difundir na saliva sem necessidade de um transporte ativo e sua concentração na saliva (~5% do total liberado no plasma) é altamente correlacionada com a fração livre no sangue.

Atualmente, a análise de cortisol salivar como marcador de atividade adrenocortical é amplamente aceita e difundida na prática clínica e pesquisa biológica. A variação na concentração de cortisol tem sido utilizada no estudo de diferenças no comportamento competitivo em primatas[22], relações entre a perda de capital no mercado de valores e estados fisiológicos[23], estados de humor e índice de desempenho[24], fobias[25], entre outras.

Enquanto o cortisol salivar é utilizado como marcador da atividade do eixo HPA, tem-se sugerido que outras substâncias na saliva podem ser indicadores da resposta do SNS ao estresse. Considerando que a secreção de proteínas na saliva é regulada por inervação simpática[20], a concentração e atividade de proteínas salivares, principalmente da alfa-amilase (sAA) e a cromogranina A salivar (CgA) tem sido utilizadas para avaliar a reatividade do SNS a estímulos adversos. A utilidade de ambas as proteínas na avaliação da atividade do SNS foi corroborada por estudos que demonstraram uma maior taxa de secreção após a administração de agentes adrenérgicos[26]e a inibição da sua secreção sob tratamento com beta-bloqueadores inclusive após o processamento de fortes emoções em sujeitos saudáveis[27].

Esporte Profissional Como Modelo de Estresse

A natureza competitiva do esporte tem a característica de induzir variações nos estados de humor. Com freqüência a prevalência de estados de humor negativos ou de excitação é maior do que a presença de estados positivos como a tranqüilidade. No esporte de rendimento, níveis consideráveis de estresse são experimentados por atletas principalmente porque do seu desempenho depende sua retribuição econômica, seu sucesso profissionalalém da sua própria satisfação pessoal. É sabido que os estados de ansiedade, excitação e de humor em geral experimentados momentos antes, durante e após uma competição, estão associados a níveis de magnitude similar na ativação do SNS[28,29] e dependem da forma como a competição é percebida. Assim, a prevalência de estados de ansiedade, medo ou agressividade está condicionada a como cada indivíduo lida com o estresse. De forma geral, uma baixa atividade do eixo HPA e do SNS está relacionada com estados de humor positivos[30] enquanto que uma maior atividade se relaciona como estados de humor negativo[24,31].

Considerando o anterior, não é difícil entender porque a competição esportiva profissional oferece um contexto excepcional na avaliação da resposta ao estresse. Além de lidar com uma série de estressores sociais e logísticos durante a preparação e na competição em si, os atletas devem manter altos níveis de desempenho (mental e físico) durante as mesmas. Devido a que o critério de avaliação durante competições esportivas é simples e bem definido e que as mesmas são altamente organizadas, a avaliação dos sujeitos é também objetiva e padronizada, permitindo um maior rigor cientifico no delineamento experimental dos estudos. Finalmente, os sujeitos são avaliados durante suas atividades do dia-a-dia e em conseqüência induz respostas reais ao estresse.

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Capitulo 2

The Cortisol Awakening Response and Perceived Upcoming Demands in Well-trained Men

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Resumo

O presente estudo investigou a variação da resposta do cortisol ao acordar (CAR) em relação com estados de humor e desempenho durante uma competição de natação profissional. Onze atletas foram examinados durantes dois dias consecutivos de competição. Em cada dia, a concentração de cortisol salivar foi determinada ao acordar, 30 e 60 minutos após acordar, imediatamente antes de aquecer para a competição e 5, 20 e 40 minutos depois da competição. O Perfil de Estados de Humor e reportes próprios de desempenho foram incluídos como instrumentos psicométricos. A CAR não foi diferente entre os dias de competição e controle e não houve relação com o desempenho em nenhum dos dois dias. No entanto, houve diferença antes de e após o evento entre os dias de competição e controle. A percepção de um desafio que irá acontecer durante o dia não necessariamente reflete a mesma magnitude de variação na CAR em sujeitos bem treinados. Isto pode ser devido a melhores mecanismos para lidar com o estresse e a fase e hora da competição em que os sujeitos foram avaliados.

Palavras Chave: Resposta do Cortisol ao Acordar; Estados de Humor; Competição; Esporte; Exercício; Saliva.

Abstract

This study examined the variation in the cortisol awakening response (CAR) in relation to mood states and performance during professional swimming competition. Eleven athletes were examined during two consecutive days of competition. On each day salivary cortisol was determined upon awakening, 30 and 60 min post awakening, immediately before warming up for competition and 5, 20 and 60 min after competition. Psychometric instruments included the Profile of Mood States and self-reports of performance. CARs did not differ between competition and non-competition days and were not related to performance on any day. However, difference was observed in the concentration of cortisol prior to and after the contest between competition and non-competition days. Perceived demands of the day ahead might not reflect the same magnitude of variation in the CAR in well-trained men. Explanations for this include better coping mechanisms and response towards the phase and time of competition.

Keywords: Cortisol Awakening Response; Mood States; Competition; Sports; Exercise; Saliva.

1.Introduction

Evidence indicates that alterations in behavior are governed by physiologic adaptive responses. In particular, the effect of adverse environments on behavior is dictated mainly, by variations in the secretion of cortisol [1]. Cortisol is released from the adrenal cortex into circulation and serves a wide range of physiological, behavioral and cognitive functions such as dampening the immune system, regulating glucose and fat metabolism, altering mood, and impairing memory formation and consolidation in response to chronic threatening circumstances [2,3].

Cortisol secretion shows a diurnal rhythm with the highest concentrations in the early morning upon awakening and decreasing concentrations over the day [4]. A distinctive feature of the circadian oscillation of cortisol, the cortisol awakening response (CAR), is a steep increase in the concentration of cortisol approximately 30 min following awakening [5,6]. The CAR is thought to reflect the sensitivity of the HPA axis to stress and other pathologies. Whereas an increased CAR has been reported in subjects with chronic stress [7,8], and work overload [9], a diminished CAR is common in patients with fatigue, burnout or exhaustion-related symptoms [10,11]. Among the explanations for such difference in cortisol profiles is the reasoning that chronic stress exposure will eventually lead to HPA axis hypo-activity, either by means of reduced biosynthesis of hormones on different levels of the HPA axis, or enhanced sensitivity to the negative feedback of cortisol [12]. However, so far no conclusive proof has been established.

Recently, it was suggested that in healthy individuals higher wakeup cortisol levels serve a metabolic purpose and so the CAR would act as a “booting”-like mechanism to help one meet the perceived demands of the upcoming day [13]. This hypothesis has gained further support when considered that the CAR could be under control not only of the hypothalamus and the pituitary, but also the

hippocampus, which is central in the formation and retrieval of memory (For review see [14] and [15]) .

Professional sports competition offers an exceptional scenario in which to assess the stress response. First, it poses a distinct psychological and physiological challenge to athletes. Not only do they cope with a wide range of organizational and social stressors both in preparation to and during competition, but also they are to sustain high levels of performance through all phases of competition. Secondly, it is highly organized and regulated and criteria of performance are clear. Therefore, collection of data and assessment of subjects is objective and standardized. Lastly, subjects are assessed in real-life environments, which elicit genuine responses to threatening situations. Here we assessed professional swimmers during two consecutive days of elite competition to further explore the proposition of the CAR as a mechanism responsive to the perceived demands of the forthcoming day. We hypothesized that 1) athletes would show an altered CAR on competition days and 2) the features of the CAR (increased or decreased when compared to non-competition days) would help predict performance later on the same day.

2. Methods

2.1. Subjects

Subjects were 11 male professional swimmers (age 21.5 ± 2.16 years) each with at least five years of experience in competition. Subjects were recruited from a team before a national swimming contest. The performance of each athlete in this competition is used to partially define his status on the team and his salary. None of the subjects smoked, or was under any kind of medication during the study. Two weeks before the competition subjects were informed of the experimental procedures and gave their written informed consent. The experimental protocol was approved by the Institutional Review Board.

2.2. Design

Subjects were asked to collect saliva samples throughout the first two days of a one-week national competition. A total of seven saliva samples were collected per day at the following times: (T1) upon awakening [while still lying in bed], (T2) 30 minutes later, (T3) one hour later [around 08:00h], (T4) immediately before warming up for competition [around 16:00h], (T5) five, (T6) 20 and (T7) 40 minutes after the competition [around 19:00h]. Subjects had approximately one hour to warm-up and all competition events took place no more than 1.5 hours after this. Subjects were provided with POMS questionnaires and labeled collection vials for saliva collection in the morning. Also, subjects were instructed to refrain from eating, drinking (anything but water) or tooth brushing during the first hour after awakening, and at least one hour before T4. Two weeks after the competition, the event was recreated at the training facilities of the swimming team for control values. Subjects performed on the same day of the week and time of the day that the real competition. Water temperature (25-28℃) was also controlled to match that of the competition. Subjects were encouraged by their coaches to perform with the same intensity and on similar swimming times during the non-competition days.

2.3. **Measures**

2.3.1. Aerobic capacity

One week before the competition VO_2max was determined during an incremental exercise test on an electronic treadmill ergometer (MicroMed Biotechnology C200) with simultaneous measurements of respiratory gas exchanges (Cortex, Metasoft 3.1) until voluntary exhaustion. Criteria to validate the test included at least two of the following parameters: plateau in VO2, respiratory exchange ratio > 1.1, and predicted maximal heart rate.

2.3.2. Saliva sampling and handling

Saliva (±2mL) was collected in collection vials with no exogenous stimulation using the guidelines proposed by Granger et al., (2007) [16]. Briefly, subjects were asked to imagine they were eating food and to slowly and gently move their jaws as if they were chewing. Saliva was drooled into the collection vials after two minutes. Samples were placed on ice, transported to the laboratory and stored frozen at -20℃ until analysis.

2.3.3. Salivary Cortisol

On the day of analysis samples were thawed and centrifuged at 1500 x g for 15 minutes. Salivary cortisol concentrations were determined employing an enzyme immunoassay (Salimetrics, State College, PA). The test used 25 µL of saliva per determination, has a lower limit of sensitivity of 0.003 µg/dL, an average intra- and inter-assay coefficient of variations of 3.5% and 5.1%, respectively. Since the collection of the first three samples was not overseen by the research staff, subjects were given clear and concise directions regarding collection procedures and the importance of punctuality. Subjects were also asked to record collection times and those providing samples deviating more than 10 minutes from the appropriate times were excluded from the analyses. All samples from each subject were assayed on the same plate and in duplicate.

2.3.4. Psychometric Instruments

Subjects completed the Profile of Mood States (POMS), immediately after collecting saliva at times T2, T4 and T6. The POMS is a 65-item questionnaire measuring tension, depression, anger, confusion, vigor and fatigue on a 5-point Likert scale [17]. At the end of each day, subjects were also asked to rate their performance based on the time and placing in the competition on a scale of 1 (poor) to 5 (outstanding). Only time was chosen as criterion of performance during non-competition days.

2.4. Statistical Analysis

Log-transformations were performed to normalize skewed variables when necessary. To verify normal distribution the Lilliefors significance correction was applied. All analyses were conducted with the log-transformed values. However, non-transformed data are shown for the sake of interpretation. Salivary cortisol levels were computed as follows: for the CAR, 1) the area under the curve with respect to ground (AUCg) and 2) the area under the curve with respect to increase (AUCi) by using the trapezoid formula [18]. Both the AUCg and AUCi were calculated for the diurnal profile of cortisol including all samples (T1-T7), in addition to the response prior to and after competition with samples T4-T7. For comparison of psychometric and performance data after log-transformations the Mann–Whitney–Wilcoxon test was used. Spearman's rank correlation coefficient was used among variables. For all analyses, significance levels was $\alpha = 0.05$. Results shown are means ± SD.

3. Results

3.1. The cortisol awakening response

As shown in Figure 1 cortisol values followed the regular awakening response with the highest levels 30 minutes after waking followed by a decrease. Areas under the curve did not differ between competition and non-competition days [Day 1; AUCg: competition mean = 59.24 ± 8.6, control mean = 54.27 ± 16.6, $t(10) = .79$, $p = 0.44$; AUCi: competition mean = 14.87 ± 14.8, control mean = 11.76 ± 10.4, $t(10) = .42$, $p = 0.67$] [Day 2; AUCg: competition mean = 65.63 ± 19.1, control mean = 46.42 ± 18.9, $t(8) = 2.27$, $p = 0.15$; AUCi: competition mean 13.56 ± 5.6, control mean 9.98 ± 8.1, $t(8) = 1.59$, $p = 0.44$]. Furthermore, no difference was seen in the AUCg when all seven cortisol samples (T1-T7) were included [Day 1; competition mean = 574.26 ± 102.9, control mean = 429.65 ± 126.9, $t(8) = 2.51$, $p = 0.055$] [Day 2; competition mean 598.57 ± 176.1, control

mean 362.90 ± 150, t(8) = 2.76, p = 0.052]. Figure 1 and 2 show the CAR and the diurnal variation of cortisol on both days of competition and control.

3.2. Pre and post-competition profile of cortisol

Figure 2 shows the pre and post-competition profile of cortisol. Areas under the curve with respect to ground were different on both days [Day 1; AUCg: competition mean 156.2 ± 31, control mean = 93.37 ± 30.8, t(10) = 1.87, p = 0.008] [Day 2; AUCg: competition mean: 142.7 ± 40.2, control mean 65.13 ± 37.7, t(10) = .53, p = 0.001]. Nevertheless, the same pattern was not observed for the AUCi [Day 1; AUCi: competition mean = 32.37 ± 23.4, control mean = 35.53 ± 25.7, t(10) = .85, p = 0.71] [Day 2; AUCi: competition mean = 12.45 ± 11.5, control mean = 11.12 ± 5.2, t(10) = 1.7, p = 0.82].

3.3. Psychometric variables and reports of performance

Table 1 shows mood variation during competition and non-competition days. Total mood disturbance differed from control at times T2 [t(10) = 4.51, p = 0.002], T4 [t(10) = 2.65, p = 0.004] and T6 [t(10) = 2.13, p = 0.33] on the first day and at T2 [t(10) = 2.54, p = 0.03] on the second day. Table 2 shows time of swimming and self-reports of performance. No difference was observed in either time or performance between days of competition and control.

3.4. Associations between the CAR and mood states

We observed negative, but not significant correlations between the CAR and total mood disturbance at T2 on both competition days [Day 1; AUCg: r(11) = -.59, p = 0.55; AUCi: r(11) = -.55, p = 0.07] [Day 2; AUCg: r(11) = -.90, p = 0.08; AUCi: r(11) = -.70, p = 0.23]. The same was seen when all seven cortisol samples (T1-T7) were considered [Day 1; AUCg: r(11) = -.78, p = 0.051; AUCi: r(11) = -.20, p =0.71] [Day 2: AUCg: r(11) = -.89, p = 0.08; AUCi: r(11) = -.30, p = 0.68]. Furthermore, we did not find any associations between mood disturbance at T6

on the first day and the CAR on the second day on both competition and non-competition days [AUCg: r(11) = .61, p = 0.28; AUCi: r(11) = .27, p = 0.74]

3.5. Associations between the CAR and performance

We did not find significant correlations between the CAR and self-reports of performance on any day [Day 1; AUCg: r(11) = .09, p = 0.77; AUCi: r(11) = -.41, p = 0.19] [Day 2; AUCg r(11) = .34, p = 0.12; AUCi: r(11) = .21, p = 0.09]. The same was seen when all seven cortisol samples (T1-T7) were considered [Day 1; AUCg: r(11) = .10, p = 0.78; AUCi: r(11) = .38, p = 0.69] [Day 2; AUCg: r(11) = -.23, p = 0.61; AUCi: r(11) = -.60, p = 0.86].

4. Discussion

Contrary to our initial hypothesis, we did not find any associations among the CAR, mood disturbance and performance. Interestingly, mood disturbance and performance were different between days of competition and control. Initially, we thought larger CARs would be seen on competition days mostly because of the psychological pressure posed by the competition. The subjects of this study, for instance, spend most of the year training for three main events, which are used by their team to define their competitive status, their salary and by the national swimming federation as trials for the selection of the national team. Since this study was conducted on one of these events, we thought that the anticipation of competition would be reflected on the morning profile of cortisol.

The postulation of the CAR as a “booting”-like mechanism [13], comes from several lines of evidence showing higher CARs on weekdays than on weekends [7,19], in individuals who regularly wake up early in the morning as well as conditions of chronic stress such as low socioeconomic status [20], work overload [9,21], and the fact that no CARs were reported when subjects wake up in the middle of the night [22]. Furthermore, from a physiological rather than a psychological perspective, there is conjectural evidence linking the CAR under

partial control of the hippocampus, which has an important role in the consolidation and activation of memory representations as well as time and spatial navigation of the self [15,23]. Thus, although hypothetical, it seems plausible that higher cortisol levels in the morning are associated with significant perceived components of novelty and workload later in the day.

However, CARs in this study did not differ between days of competition and control even though the swimming contest was clearly of major importance to the subjects. It may be argued that experience in competition and socioeconomic status played an important factor on the psychological response to challenge. All subjects had had frequent participation in national and international contests for the last five years prior to this study. In addition, they come from a medium and high socioeconomic status, thus psychological duress related to the financial consequences of performance would be little. In this respect, a thorough study on the diurnal profile of cortisol secretion related to ballroom dancing competition reported that 1) the response to competition does not seem to adjust with experience, so both novice and veteran athletes show a significant increase in cortisol prior to competing and 2) among the psychological variables responsible for the rise in cortisol before a competition, the threat to the social-self is one of the most potent factors to affect the HPA axis [24]. Furthermore, moderately high negative correlations were observed between mood states 30 min post awakening (T2) and the CAR as well as differences between mood states on days of competition and control. This indicates that the contest was in fact perceived as a strong psychological stimulus and that the lack of association between the CAR and performance was most likely not due to chance.

Evidence of the CAR in healthy individuals as a signature of a burdensome day is still controversial. In fact, while a significant body of research has associated alterations in the CAR with stress-related conditions, few studies have been directed to explicitly test this hypothesis. For instance, prior-day feelings of loneliness, sadness and threat have predicted increased CARs the next morning. Decreased CARs have also been associated with feelings of tension and anger

later the same day in older adults [13]. On the other hand, no associations were reported between the CAR and same-day anticipations of a busier day in young women [25]. Our results add to this body of evidence by showing that distinct psychological and physiological challenges later in the day do not necessarily reflect the same magnitude of variation in the CAR in well-trained young men. Interestingly, there was an increase in the diurnal profile of cortisol from the first to the second day of competition to the point of almost reaching statistical significance ($p = 0.052$). The format of this kind of competitions consists of eliminatory rounds, so athletes are faced against bigger challenges (faster opponents) with each subsequent day of competition. Thus, it might have been possible that the CAR showed responses of greater extent on the final stages of competition as both the opponents and the consequences of winning or losing were also of major importance. However, until this finding is replicated including week-long assessments of cortisol, we have elected to avoid speculation about its meaning.

On the other hand, the cortisol profile observed immediately before and after swimming was notably higher on competition days. Such rise in the concentration of cortisol prior to sports competition has been attributed to the intensity of physical exercise [26], an improved anaerobic metabolism [27] and the expectation of competition itself [24,28]. The fact that higher concentrations of cortisol were seen on competition days and that no difference in swimming time or performance between days of competition and control were present lead us to believe that the difference in cortisol was due to psychological and not physiological factors. Although it may be argued that it is usually difficult to achieve similar levels of performance during training due to the lack of pressure and motivation, it is also unlikely that little variation in intensity resulted in such different profiles of cortisol both before and after exercising [24]. The increase in cortisol on competition days is more likely related to mood states variations and coping mechanisms. Previous research indicates that higher concentrations of cortisol both prior to and after competition are associated with motivation [29], competitiveness and mental preparation in males, social bonding in females [28]

and with high insecurity indexes and poor performance in fighters [30]. We did not find any correlations between concentrations of cortisol prior to swimming and performance. However, higher concentrations of cortisol were associated with a higher score of mood disturbance on the first day of competition.

It is noteworthy to mention that the time at which the contest took place might have influenced the dynamics of the CAR. Athletes were to start warming-up at 16:00h and all contests took place approximately two hours later. Considering that they were to wake up at 07:00h, it is possible that subjects only became fully involved in competition early in the afternoon. Well-trained subjects have previously shown lower cortisol responses to a series of adverse psychological stimuli [31]. Thus, early in the morning they might have coped better with the pressure of having to compete and only showed significant variations in the concentration of cortisol once they perceived the contest was imminent. Further studies should examine whether the time at which athletes are to compete has some effect on the profile of the CAR.

Limitations to this study include the modest sample size and saliva collection procedures during the morning. Nevertheless, our sample was also very homogeneous. The subjects in this study were all male, within a narrow age range, had a very high and similar level of performance in addition to an extensive experience in professional competition. Furthermore, we included a comprehensive diurnal profile of cortisol with a total of seven samples per day, which allowed us to observe timely variations in the concentration of cortisol both before and after the competition. Finally, baseline values were obtained from the same subjects in a carefully recreated event that matched day-of-the-week and time-of-the-day assessments. Several other studies on the associations of the CAR and mood states in humans have used similar or even smaller samples and some found corresponding results to our study [24,25,32-37]. Thus, we believe the assumption of a null hypothesis in this study is valid and little, if any, variation would have been seen with a larger sample. Moreover, although including a larger sample would be desirable, experimental designs often interfere with

training sessions or competition events further complicating the evaluation of the stress response in this particular population. Regarding collection of saliva samples in the morning, the subjects in this study together with the coaching and two members of the research staff were housed at the same hotel during the competition. Eating times were strictly controlled by the coaching staff and breakfast was scheduled promptly after T3. All collection vials were collected by the research staff at approximately 08:00 immediately after T3, thus subjects had corresponding waking times. In addition to this, samples collected more than 10 minutes deviating from the scheduled times were not considered in the analysis. Consequently, we believe that the limitations in our study are counterbalanced by the uniformity of the sample, the thorough design and the caution taken when collection of saliva was not overseen.

5. Conclusions

Our results indicate that distinct psychological and physiological challenges later in the day do not necessarily reflect the same extent of variation in the CAR in well-trained men. Likewise, the features of the CAR were not associated with performance or mood disturbance. Since differences in the concentration of cortisol were seen only prior to and after exercising and the swimming contests took place late in the afternoon, examining variations in the CAR when the competition is held in the morning would help to explain the hypothesis of the CAR as a mechanism subjected to the perceived demands of the day ahead.

Conflict of interest

None declared.

Role of the Funding Source

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Table 1. Demographic features and mood disturbance scores of subjects during competition and non-competition days. For mood disturbance scores, the upper line (in bold) and lower line indicate competition and non-competition days, respectively. Values are means(SD).

Age	21.55 (2.16) years											
BMI	22.7 (2.5)											
VO_2 Max	52.7 (3.28) ml/kg.min											
Competition Experience	8.7 (2.8) years											
	Day 1						**Day 2**					
POMS Subscale Scores	**T2**	**p**	**T4**	**p**	**T6**	**p**	**T2**	**p**	**T4**	**p**	**T6**	**p**
Tension – Anxiety	**8.8 (4.0)**	0.067	**13.3 (6.4)**	0.015	**10.3 (6.7)**	0.036	**9.8 (4.2)**	0.103	**10.0 (4.6)**	0.180	**7.6 (6.5)**	0.127
	3.3 (2.6)		3.8 (1.4)		3.0 (0.6)		4.0 (3.8)		6.0 (2.5)		2.4 (0.5)	
Depression	**3.5 (2.3)**	0.075	**3.6 (2.0)**	0.008	**13.8 (10.8)**	0.079	**4.0 (1.8)**	0.140	**2.2 (1.6)**	0.070	**7.4 (8.6)**	0.330
	1.5 (2.0)		1.3 (1.5)		5.5 (6.3)		1.8 (2.4)		0.4 (0.8)		4.2 (3.6)	
Anger – Hostility	**7.8 (2.9)**	0.155	**9.8 (5.1)**	0.006	**16.6 (12.4)**	0.043	**7.6 (4.8)**	0.152	**5.2 (4.7)**	0.303	**9.2 (10.8)**	0.409
	4.5 (5.6)		4.3 (5.0)		5.6 (5.0)		2.2 (4.3)		2.6 (3.7)		4.2 (2.8)	
Vigor – Activity	**17.6 (5.2)**	0.556	**19.5 (5.2)**	0.462	**12.6 (5.5)**	0.448	**18.2 (4.1)**	0.919	**18.8 (6.2)**	0.255	**15.0 (7.3)**	0.767
	15.6 (5.7)		17.5 (2.8)		11.8 (4.3)		17.8 (5.0)		20.6 (4.2)		16.0 (8.2)	
Fatigue	**6.6 (3.6)**	0.175	**5.5 (4.7)**	0.135	**18.1 (6.7)**	0.022	**5.4 (3.2)**	0.740	**4.2 (4.7)**	0.412	**12.4 (11.1)**	0.125
	4.1 (3.6)		3.5 (3.9)		12.5 (7.6)		4.8 (4.6)		2.6 (2.7)		8.8 (8.4)	
Confusion-Bewilderment	**10.0 (2.6)**	0.007	**10.0 (3.3)**	0.016	**9.0 (3.8)**	0.048	**9.2 (3.2)**	0.073	**8.4 (5.3)**	0.186	**7.4 (5.7)**	0.377
	3.6 (1.3)		5.1 (1.7)		5.8 (3.4)		4.0 (2.3)		4.2 (1.3)		4.8 (2.3)	
Total Mood Disturbance	**21.0 (8.9)**	0.002	**21.5 (13.4)**	0.004	**52.3 (31.8)**	0.033	**17.2 (11.6)**	0.030	**15.0 (15.30)**	0.089	**33.6 (31.3)**	0.113
	3.1 (3.3)		4.3 (8.4)		20.0 (20.3)		3.0 (4.7)		0.8 (1.7)		16.1 (11.0)	

* For mood disturbance scores, the upper line (in bold) and lower line indicate days of competition and control, respectively. Values are means (SD).

Table 2. Time of swimming and self-reports of performance during days of competition and control.

	Day 1	p	Day 2	p
Time of swimming (seconds)	**53.16 (2.02)**	0.76	**52.46 (1.06)**	0.085
	53.43 (1.88)		53.62 (1.71)	
Performance	**3.63 (0.67)**	0.11	**3.71 (0.64)**	0.074
	3.18 (0.60)		3.27 (0.46)	

* The upper line (in bold) and lower line indicate competition and control, respectively. Values are means (SD).

Figure 1

Morning profile of salivary cortisol at awakening, 30 ad 60 minutes later during competition and non-competition days. Values are means and error bars indicate SD.

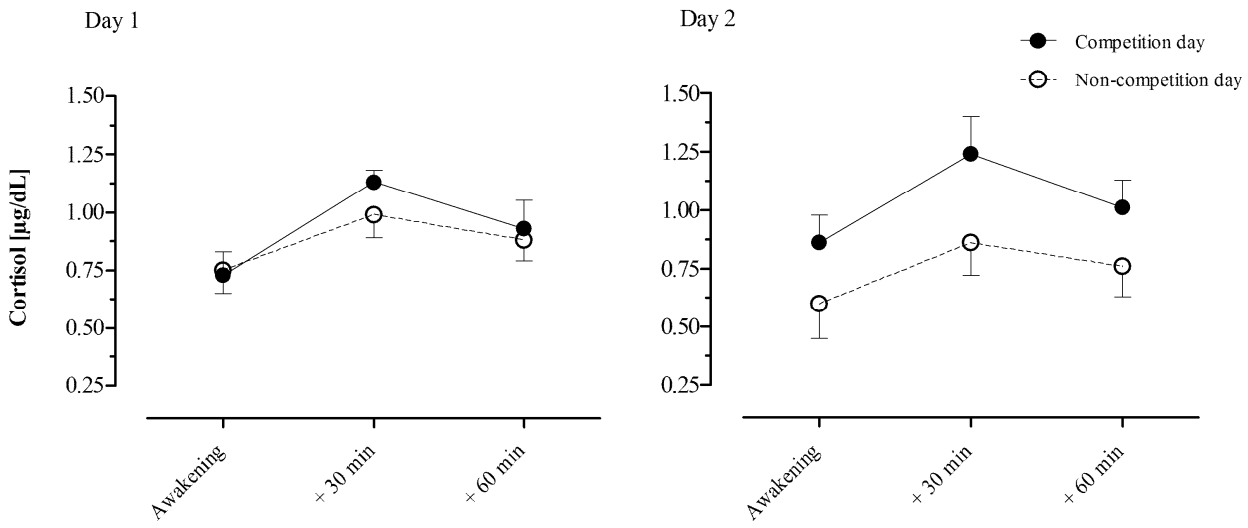
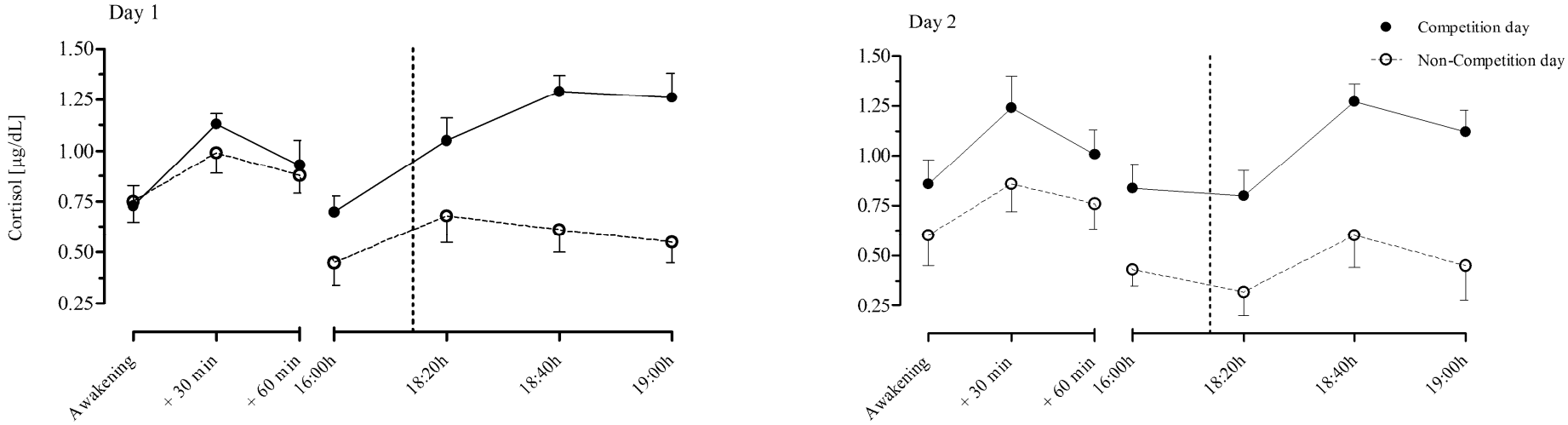


Figure 2

Diurnal profile of salivary cortisol during competition and non-competition days. The dotted vertical line indicates time of swimming. Values are means and error bars indicate SD.



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Capitulo 3

Differential Changes in Salivary Markers of Autonomic Activity in Response to Elite Competition

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Resumo

O presente estudo investigou a resposta de proteína total na saliva (TP) alfa-amilase (sAA) e cromogranina A (CgA) à competição esportiva e sua relação com estados de humor. Onze atletas profissionais foram examinados durante o primeiro dia de um evento nacional em natação e durante um evento simulado num dia de treinamento no mesmo dia da semana e no mesmo horário que a competição real. A concentração de TP foi determinada pelo método de Bradford e a de sAA e CgA por western blotting ao acordar, 30 e 60 minutos depois, imediatamente antes de aquecer para o evento e 5, 20 e 40 minutos depois do mesmo. A escala de estado de humor PANAS-X (Positive Affect and Negative Affect Schedule) foi incluída como instrumento psicométrico. A concentração de TP, sAA e CgA foi diferente dos dias de controle unicamente antes e 5 minutos após a competição. Não houve diferença na área sob a curva do perfil diurno de cada marcador entre os dias de competição e controle. TP e CgA tiveram uma resposta similar à competição do que sAA. Isto pode ser atribuído aos mecanismos de secreção de proteína na saliva quando a coleta da mesma é feita sem estimulação exógena. Estímulos psicológicos adversos parecem alterar o ritmo regular de secreção destes marcadores só momentos prévios e posteriores a situações estressantes.

Palavras Chave: Proteína Total, Alfa-amilase, Cromogranina A, Saliva, Competição, Exercise, Psico-fisiologia, Stresse.

Abstract

Objective: We investigated the response of salivary total protein (TP), alpha-amylase (sAA) and chromogranin A (CgA) to sporting competition and their relation with positive and negative affect.

Methods: Eleven professional swimmers were examined during the first day of a national contest and on a recreated event that matched time-of-the-day and day-of-the-week assessments two weeks later. Total protein was determined by the Bradford method and sAA and CgA by western blotting upon awakening, 30 and 60 min post awakening, immediately before warming up for competition and 5, 20 and 60 min after competition. Psychometric instruments included the Positive Affect and Negative Affect Schedule - X (PANAS-X).

Results: The concentrations of TP, sAA and CgA differed from controls only prior to and 5 min after the event. We observed associations between higher negative affect scores with higher levels of TP, sAA and CgA prior to the event on the competition day. Areas under the curve did not differ from controls for TP, sAA or CgA.

Conclusion: TP and CgA showed a similar reactivity to sporting competition than sAA, which may be attributed to the mechanisms responsible for protein secretion into saliva when collection is performed with no exogenous stimulation. Strong adverse psychological stimuli only seem to override the regular rhythm of salivary proteins moments before and after stressful situations.

Keywords: Alpha-amylase; Chromogranin A; Total Protein; Saliva; Competition; Exercise; Psychophysiology; Stress.

Introduction

The stress response is subserved by components in the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenocortical (HPA) axis. The ANS stimulates the adrenal medulla to produce catecholamines whereas glucocorticoids are the final effectors of the HPA axis (Tsigos & Chrousos, 2002). More than a decade ago, salivary alpha-amylase (sAA) was suggested as a surrogate marker for autonomic activity given that its release into saliva is elicited by stimulation of the salivary glands by sympathetic and parasympathetic nerves (Rohleder & U. M. Nater, 2009). Furthermore, following research demonstrated that beta-adrenergic agonists are capable of stimulating sAA release without increasing salivary flow (Ehlert, Erni, Hebisch, & U. Nater, 2006) and beta-blocking agents inhibit sAA secretion in response to psychological challenges (A. van Stegeren, Rohleder, Everaerd, & O. T. Wolf, 2006). This pattern of evidence has resulted in the incorporation of sAA into a wide series of behavioural studies in which the sAA response to adverse environments has been measured and even successfully applied in clinical (Uesato et al., 2010) and military settings (Cosenzo, Fatkin, & Patton, 2007).

Chromogranin A (CgA), on the other hand, is co-stored and co-released with catecholamines from secretory vesicles in the adrenal medulla and post-ganglionic sympathetic axons (Zhang et al., 2006). Chromogranin A is also produced by the submandibular gland and secreted into saliva under autonomic control and presents antifungal and antimicrobial properties (Lugardon et al., 2000; Saruta et al., 2005; Strub et al., 1996). Salivary CgA has received some attention as a marker of psychological stress and similar responses to adverse psychological stimuli than sAA have been reported. In particular, higher concentrations of CgA were present after lectures to university graduates in young professors (Filaire, Dreux, Massart, Nourrit, et al., 2009b), after moderate exercise (Allgrove, Gomes, Hough, & Gleeson, 2008), and following cognitive assessments (Kanamaru, Kikukawa, & Shimamura, 2006).

Although sAA and CgA are secreted into saliva from different glands, the parotid and submandibular glands, respectively, the secretion of both proteins into saliva from these glands is under autonomic control. In addition to the parotid and submandibular glands, the sublingual and numerous minor glands also contribute to the secretion and composition of whole mouth saliva. Essentially, parasympathetic input to salivary glands produces most of the fluid in saliva whereas sympathetic stimulation results in protein secretion (Proctor & Carpenter, 2007). Although this view is somewhat simplistic and exceptions to such pattern of saliva secretion exist, one could expect to observe similar responses of both sAA and CgA to adverse environments. By the same token, it could be speculated that not only sAA and CgA but also salivary total protein (TP) showed a similar response to hostile stimuli. However, most psychophysiological research on salivary correlates of autonomic activity has focused on sAA and missing are reports that enable us to speculate whether the variation in the composition of saliva in anticipation of and in response to psychological and physiological demands is particular to the dynamics of single markers or dependent on the mechanisms responsible for their secretion into saliva.

Professional sports competition offers a unique scenario in which to assess the response to stress. It involves social comparison and evaluation and is a significant source of pressure for athletes (Gucciardi & Dimmock, 2008). Secondly, it is highly organized and criteria of performance are clear. Therefore, collection of data and assessment of subjects is objective and standardized. Moreover, since subjects are assessed in real-life environments, authentic responses to adverse stimuli are observed. It is well established that competition induces sympathetic arousal both in anticipation of and in response to the event (Baron et al., 1992; Kraemer et al., 2001). Such variation in autonomic activity is related to the perceived nature and demands posed by the competition. Thus, subjects may experience feelings from worry and fear to vigour and aggressiveness depending on how they cope with stress. To a large extent, a lower reactivity of the HPA axis and the ANS system has been associated with positive affect (Dockray & Steptoe, 2010) whereas a higher reactivity is related to

negative affect (Adam, Hawkley, Kudielka, & Cacioppo, 2006; Rohleder & U. M. Nater, 2009).

In this study, we examined the variation in salivary TP, sAA and CgA in professional swimmers during a national contest and on a regular training day. In theory, a higher autonomic drive would be present on the competition rather than the non-competition day due to higher components of anxiety and pressure. Thus, we hypothesized that 1) higher concentrations of TP, sAA and CgA would be observed on the competition relative to the control day and this would be associated with higher scores in mood disturbance, and 2) little difference amongst the dynamics of TP, sAA and CgA would be present within the competition or the control day.

Methods

Subjects. Subjects were 11 male swimmers (age 21.5 ± 2.16 years) each with at least five years of experience as a professional athlete. Subjects were recruited from a team before a national swimming competition. The performance of each athlete in these competitions is used to partially define his status on the team and his salary. None of the subjects smoked, or was under any kind of medication during the study. Two weeks before the competition subjects were informed of the experimental procedures and gave their written informed consent. The experimental protocol was approved by the Institutional Review Board.

Experimental design. Subjects were asked to collect saliva samples throughout the first day of a one-week national competition. A total of seven saliva samples were collected at the following times: (T1) upon awakening [while still lying in bed], (T2) 30 minutes later, (T3) one hour later [around 0800h], (T4) immediately before warming up for competition [around 1600h], (T5) five, (T6) 20 and (T7) 40 minutes after the competition [around 1900h]. Morning samples (T1-T3) were included to explore probable relations between the awakening response of salivary proteins and mood disturbance induced by competition.

Subjects were instructed to refrain from eating, drinking (anything but water) or tooth brushing during the first hour after awakening, or at least one hour before T4. Subjects were also asked to refrain from alcohol or caffeinated beverages at least 24 hours prior days of saliva collection. Two weeks after the competition, the event was recreated at the training facilities of the swimming team for control values. Subjects performed on the same day of the week and time of the day that the real competition. Water temperature (25-28℃) was also controlled to match that of the competition. Subjects were encouraged by their coaches to perform with the same intensity and on similar swimming times during the control day.

Measures

Aerobic capacity. One week before the competition VO_2max was determined during an incremental exercise test on an electronic treadmill ergometer (MicroMed Biotechnology C200) with simultaneous measurements of respiratory gas exchanges (Cortex, Metasoft 3.1) until voluntary exhaustion. Criteria to validate the test included at least two of the following parameters: plateau in VO2, respiratory exchange ratio > 1.1, and predicted maximal heart rate.

Saliva sampling and handling. Whole mouth saliva was collected into collection vials with no exogenous stimulation using the guidelines proposed by Granger and colleagues (2007). Briefly, subjects were asked to imagine they were eating food and to slowly and gently move their jaws as if they were chewing. Saliva was drooled into pre-weighted conical polypropylene tubes after two minutes. Immediately after the event (before T5) subjects were given 70 ml of distilled water to wash their mouths. Subjects were asked to spit the water and to swallow in order to empty the mouth before saliva was collected. Samples were placed on ice, transported to the laboratory and stored frozen at -20℃ until analysis.

Western blotting for sAA and CgA. On the day of analysis samples were thawed and centrifuged at 3000 rpm for 15 minutes. Concentration of total protein in samples was determined using the Bradford method (Bradford, 1976). All samples from

each subject were assayed on the same plate in duplicate. In order to avoid possible effects of salivary flow rate on the concentration of proteins, especially after exercising (dehydration), ten micrograms of total protein from each sample were denatured under reducing conditions and applied on 5–20% SDS–polyacrylamide gradient gels. Proteins were separated and then transferred onto nitrocellulose membranes in transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH, pH 7.8-8.4) for two hours at 200mA and 4℃. Protein transfer was confirmed by visualization with Ponceau. Membranes were blocked for 4 hours at 4℃ in blocking buffer (5% non-fat dry milk in PBS w/v). Membranes were then incubated overnight at 4℃ with purified polyclonal rabbit anti-human sAA (dilution 1:5000) and mouse monoclonal anti-human CgA (dilution 1:1000), respectively, and subsequently incubated with secondary antibodies for two hours. After incubations with specific primary and then secondary antibodies, labelled proteins were detected using ECL reagents and exposing the developed blots to GE Healthcare films. Densitometrical analysis of the spots was performed using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) by a researcher blinded to the experimental groups (competition vs. non-competition day). The area in pixels of each spot was determined in triplicate and means were used for statistical analyses.

Production of the antibody for sAA. One rabbit was immunized by a subcutaneous injection of 500ug/mL of human sAA and Freund's complete adjuvant followed by two booster injections of 250µg/mL with Freund's incomplete adjuvant two weeks apart. Blood was collected from the ear vein one week before the first administration, one week after the first two administrations and two weeks after the last administration. Serum levels of anti-human sAA were determined by ELISA and the specificity of the purified antibody was verified by Western Blotting. The rabbit was kept in constant temperature of 22 ± 2 ℃ in 12:12 dark–light cycle with constant access to chow and water. All animal procedures were approved by the Animal Ethics Committee. The anti-human CgA antibody was purchased from Millipore (Temecula, CA).

Psychometric instruments. Subjects completed the Positive and Negative Affect Schedule-X (PANAS-X), immediately after collecting saliva at times T2, T4 and

T6. The PANAS-X is a self-report measure of positive and negative affect composed of eleven separate 5-point Likert scales in which subjects rate the extent to which they are currently experiencing each emotion (Watson & Clark, 1994).

Statistical Analyses

Data were tested for normality using the Kolmogorov–Smirnov test prior to analyses. No transformations were necessary for any of the variables. The concentration of TP, sAA and CgA at each sampling time was averaged and compared between competition and control days using paired t tests. Psychometric scores were also compared using paired t tests. The area under the curve of the diurnal profile of TP, sAA and CgA was calculated using the trapezoid formula (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). For all analyses, significance levels was $\alpha = 0.05$. Results shown are means (SD) unless otherwise stated.

Results

Main characteristics of subjects. Table 1 shows main characteristics of subjects. Overall, subjects were within a narrow age range, had extensive experience as professional athletes and a high aerobic capacity.

Averaged concentrations of salivary markers of sympathetic activity. Figures 1 and 2 show the diurnal profile of TP, sAA and CgA in the morning as well as prior to and after competition. In general, all markers showed a similar pattern with a distinct decrease 30 min post-awakening with following increasing concentrations 60 min post awakening. We did not observe significant differences in the concentrations of TP, sAA and CgA in the morning period between competition and control days. However, the concentration of the three markers was different prior to and immediately after exercising. Means did not differ between competition and control days at T6 or T7 for any of the variables (Table 2).

Diurnal profile of salivary markers of sympathetic activity during competition. Here we set out to investigate whether the diurnal course of TP, sAA and CgA was different between competition and control days. Areas under the curve with

respect to ground (AUCg) [TP t(4) =0.95, p =0.39; sAA t(6) =1.11, p=0.31; CgA t(4)= 0.48, p=0.65] and with respect to increase (AUCi) [TP t(8) =1.4, p=0.17; sAA t(4) = 4.2, p=0.07; CgA t(4)= 1.53, p=0.19] did not differ for any variable between competition and control days.

Positive and negative affect scores. Table 1 shows positive and negative affect scores throughout competition and control days. Overall, we found different and higher negative scores in the morning and prior to competition whereas different but lower positive scores prior to and after competition than on the control day. Cronbach's alpha value was 0.84 and 0.86 for the Positive and Negative Affect scales, respectively.

Discussion

In order to understand the dynamics of salivary markers of autonomic activity prior to and after competition, the mechanisms of protein secretion into saliva must be considered. In essence, saliva is constantly secreted at a basal rate from three pairs of major glands (parotid, submandibular and sublingual) and from numerous minor glands spread over the oral mucosa. However, copious salivary secretion is induced upon sensory and mechanical stimuli by autonomic control to the salivary glands (Mese & Matsuo, 2007). Whereas parasympathetic nerves are mainly responsible for the secretion of water and electrolytes, sympathetic nerves are predominantly responsible for the secretion of proteins. Exceptions to this include, amongst others, the secretion of sAA from glands mainly stimulated by parasympathetic nerves, such as the palate and the sublingual glands (Jos A Bosch, Veerman, de Geus, & Proctor, 2011), and increased salivary secretion of immunoglobulin A as a result of stimuli from both sympathetic and parasympathetic nerves to plasma cells (Proctor & Carpenter, 2007). Nevertheless, evidence suggests that the rate of protein secretion into saliva by sympathetic stimuli is superimposed upon parasympathetic stimulation when glands are simultaneously innervated (Proctor & Carpenter, 2007). In addition to this, it is thought that the neural pathways that innervate salivary glands are under control of higher centres of the brains such as the cerebral cortex and the limbic centre. Consequently,

the composition and volume of saliva is responsive not only to sensory and mechanical stimuli, but also emotional states e.g., stress (Mese & Matsuo, 2007).

In line with our original hypothesis, we found a marked elevation of TP, sAA and CgA prior to and after competition. However, in contrast to our expectations we did not observe differential changes in the concentration of salivary proteins upon awakening and 60 min later even though higher negative affect scores were reported. This finding might be interpreted as follows. First, given the extensive experience of subjects in competition, they may have engaged mentally in competition only later in the day as the contest was approaching. Secondly, the magnitude of the feelings experienced early in the morning, although different from the control day, was not sufficiently intense to evoke significant sympathetic arousal.

A considerable body of research has been dedicated to assess the response of markers of autonomic activity both in anticipation of and after adverse environments. Our findings are consistent with some (J A Bosch, H. S. Brand, Ligtenberg, Bermond, et al., 1996a; Chatterton, Vogelsong, Lu, & Hudgens, 1997; Kanamaru et al., 2006; U. M. Nater et al., 2005), but not all (Kivlighan & Granger, 2006), studies that have reported significant increases in the activity of sAA and the concentration of CgA before academic and social evaluations and parachute jumping but not in collegiate competition. The subjects in our study were assessed during a national swimming contest. All subjects were professional athletes and their performance in the competition had significant consequences on their salary and competitive status within their team. Thus, and although speculative, it seems that in situations in which the outcome of the challenge poses serious threats to the self as those seen in contexts of assessment of job performance or physical danger, a rise in the activity and concentration of these proteins before taking part in stressful situations could be expected.

In contrast to CgA, the increase in the activity of sAA after challenging environments seems to be unequivocal (Kivlighan & Granger, 2006; U. M. Nater et al., 2005; A. H. van Stegeren, O. T. Wolf, & Kindt, 2008). Whereas there is evidence that the concentration

of CgA increases after strong negative stimuli (Allgrove et al., 2008; Edith Filaire, B. Dreux, Massart, Nourrit, et al., 2009b; Kanamaru et al., 2006) not all studies have been able to reproduce such results (Wagner et al., 2010; T. Yamakoshi et al., 2009). It has been proposed that catestain, which is co-secreted with CgA, inhibits further CgA release by acting as a non-competitive cholinergic antagonist and therefore as a feedback mechanism for sympathoadrenal activity (T. Yamakoshi et al., 2009). However, evidence for this mechanism exists only for the release of catecholamines from the adrenal medulla and not from the submandibular gland. On the other hand, it may be argued that in our study such rise in TP, sAA and CgA would be evident due to a higher sympathetic drive associated with exercise. Nonetheless, subjects performed within similar swimming times on the control day and it is unlikely that little difference in intensity resulted in such divergent profiles in the concentration of proteins prior to and immediately after the contest. Furthermore, we also found higher negative and lower positive affect scores relative to the control day before the contest. Thus, we believe the variation in the concentrations of proteins between days was in fact due to psychological factors associated with the threat to the self posed by the competition. Future studies would certainly benefit from more precise parameters of exercise intensity such as lactate in order to better explain the divergence in the profile of proteins when physical challenges are chosen to assess the response to stress (Rohleder, Beulen, Chen, J. M. Wolf, & Kirschbaum, 2007).

Salivary alpha-amylase is a digestive enzyme that hydrolyzes starch to glucose and maltose (Scannapieco, Torres, & Levine, 1993). It has been suggested that increases in the activity of sAA after physiological challenges may be associated with a more efficient replenishment of energy storages (Kivlighan & Granger, 2006). Considering the adaptive response to stress (Motzer & Hertig, 2004) it makes sense that not only digestive proteins (sAA), but also immune proteins (CgA) increased immediately after acute exposure to physiological and psychological threats. However, it is worth mentioning that when exercise is chosen to assess the stress response special attention must be paid to salivary flow after exercise since dehydration can strongly

influence the concentration of salivary proteins. While lower rates of salivary flow have only been reported after prolonged exercise (<30 min) (Walsh, Montague, Callow, & Rowlands, 2004) and in our study subjects were assessed after contests that took no more than two minutes, we addressed this issue by determining the concentration of both sAA and CgA using the same quantity of protein from each sample (10µg) independently of the volume of saliva collected as previously suggested (de Oliveira et al., 2010). Thus, when controlled for protein concentration, our results are in line with recent research that shows peak levels of sAA approximately 5-10 min after the adverse stimuli and a subsequent decrease reaching baseline levels 20-30 min later (Allgrove et al., 2008; Edith Filaire, B. Dreux, Massart, Nourrit, et al., 2009b; Kivlighan & Granger, 2006) adding to the proposition of Kivlighan and colleagues of an increased concentration of digestive and immune salivary proteins in response to acute adverse demands.

Several of the findings of our study are novel. First, to the best of our knowledge this is the first demonstration of the reactivity of TP and CgA to professional competition. Interestingly, both TP and CgA showed a similar response to competition than sAA with differential changes in anticipation of and after the contest. This may have important repercussions in psychophysiological research. Since the proposition of sAA as surrogate marker of sympathetic activity a significant series of studies has assessed the variation in its activity under different conditions. However, from a laboratorial standpoint, determining the concentration of TP is faster, cheaper and more practical than traditional kinetic or immune- assays. In this respect, TP has been successfully applied to monitor exercise intensity and hydration status (Bortolini et al., 2009; Neil P Walsh, J. C. Montague, Callow, et al., 2004). Clearly, further research needs to corroborate whether the pattern of secretion of sAA and CgA is similar than TP in other adverse environments. Secondly, it appears that the dynamics of such markers are not strongly associated with variations in negative and positive emotions. Probably, other scales with predominant components of tension, anxiety and excitement as seen in sympathetic arousal would have been more appropriate to detect the same magnitude

of variation, if any, between negative and positive affect and salivary proteins. Finally, distinct challenging psychological stimuli represented here by professional competition only seem to override the regular rhythm of salivary proteins prior to and immediately after the contest. In accordance with the morning profile of our data are previous studies that have reported diurnal rhythms of sAA and CgA with nadir concentrations early in the morning and an awakening response with a steep decrease 30 min after awakening (Den, Toda, Ohira, & Morimoto, 2011; J. Strahler, Berndt, Kirschbaum, & Rohleder, 2010).

On the other hand, the results of this study have to be interpreted in light of some methodological limitations. First, we controlled the concentration of protein and flow rate only for sAA and CgA. Recent data suggests that the activity of sAA is not dependent of secretion rate (Rohleder, J. M. Wolf, Maldonado, & Kirschbaum, 2006) and that only prolonged exercise causes significant loss of water (Neil P Walsh, J. C. Montague, Callow, et al., 2004). Although in our study subjects took no more than 2 min to finish the contest, future studies should examine whether exercise of short duration and high intensity has some effect on TP secretion rate. Secondly, we used a sample of subjects of modest size. We chose to assess the response to stress in sporting competition because it poses significant psychological and physiological demands to subjects. Also, it elicits genuine responses to stress since subjects are assessed in real-life situations. It is usually difficult to include a larger sample size in experimental studies because there are few professional teams with larger and homogenous groups of athletes. In addition, experimental designs often interfere with training sessions or competition events. To compensate for sample size, we designed a comprehensive protocol that allowed us to observe timely variations in the concentrations of protein prior to and in response to the task. Further, subjects were all male, within a narrow age range, had similar levels of performance and extensive experience in competition. Additionally, baseline values were obtained from the same subjects in a carefully recreated event that matched time-of-the-day and day-of-the-week assessments. Several other studies on the variation in salivary constituents in response to stress and exercise have

included similar if not smaller samples (Bullock, Cox, Martin, & Marino, 2009; Filaire, Alix, Ferrand, & Verger, 2009; Fortes & Whitham, 2011; U. M. Nater et al., 2005; Noto, Kudo, & Hirota, 2010; Oliver, Laing, Wilson, Bilzon, & Walsh, 2008; Ship & Fischer, 1999; K. Strahler, Ehrlenspiel, Heene, & R. Brand, 2010) and some have reported equivalent results than our study. Thus, although it would be desirable to work with a larger group, we believe based on previous research, that our experimental design and the characteristics of the subjects that little, if any, difference in the dynamics of TP, sAA and CgA would have been observed with a larger population.

Conclusions

Taken together, these data indicate that TP, sAA and CgA show a similar pattern of reactivity towards professional competition. Also, the dynamics of such markers do not strictly reflect alterations in negative and positive emotions. Finally, changes in the concentration of sAA, CgA and TP become apparent only moments before and after taking part in competition.

Conflict of interest

None declared.

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Table 1

Main characteristics of subjects and positive and negative affect scores during competition and control days.

Values in the upper line (bold) indicate scores during competition whereas values in the lower line indicate scores during the control day. Values are means (SD).

Age	21.55 (2.16) years		
BMI	22.7 (2.5)		
VO_2 Max	52.7 (3.28) ml/kg.min		
Competition experience	8.7 (2.8) years		
Collection Times	**T2**	**T4**	**T6**
Negative Affect	**18.6 (3.67)***	**21.6 (6.93)***	**20.0 (7.13)**
	10.8 (1.03)	12.0 (1.70)	15.60 (4.50)
Positive Affect	**28.5 (5.64)**	**29.8 (4.84)**	**23.1 (3.28)***
	31.0 (9.17)	38.0 (6.16)	30.7 (3.16)

* Significantly different from controls at $p<0.05$.

Table 2

Variation in sAA, CgA and TP during Competition and Control Days.

Values in the upper line (bold) indicate competition whereas the lower line indicates the control day. Values are means (SD).

Collection Times	T1	T2	T3	T4	T5	T6	T7
sAA (pixel density)	**9377 (2195)**	**8711 (1620)**	**9455 (2102)**	**19420 (2073)***	**22274 (2285)***	**16493 (4233)**	**20042 (2917)**
	5789 (1495)	5984 (1097)	6985 (2408)	13721 (1206)	15571 (2245)	14222 (4577)	16478 (3069)
CgA (pixel density)	**8785 (4114)**	**6693 (3985)**	**8239 (2341)**	**25190 (3564)***	**28592 (4382)***	**14973 (2349)**	**10994 (3997)**
	3117 (2994)	2134 (1998)	5505 (2145)	14955 (2495)	16698 (3489)	16376 (4981)	16763 (3467)
TP (µg/µL)	**1.15 (0.05)**	**0.95 (0.10)**	**1.00 (0.09)**	**1.35 (0.09)***	**1.42 (0.07)***	**1.21 (0.09)**	**1.10 (0.11)**
	1.28 (0.07)	0.79 (0.09)	1.19 (0.11)	1.06 (0.07)	1.14 (0.06)	1.28 (0.10)	1.23 (0.10)

* Significantly different from controls at $p<0.05$.

Figures

*Figure 1.*Diurnal Profile of sAA and CgA during Days of Competition and Control.
A shows representative results from sAA and CgA. B summarizes the quantitative results of the variation in the concentration of sAA and CgA during days of competition and control. The dotted vertical line indicates time of swimming.
Values are means and error bars indicate SD.

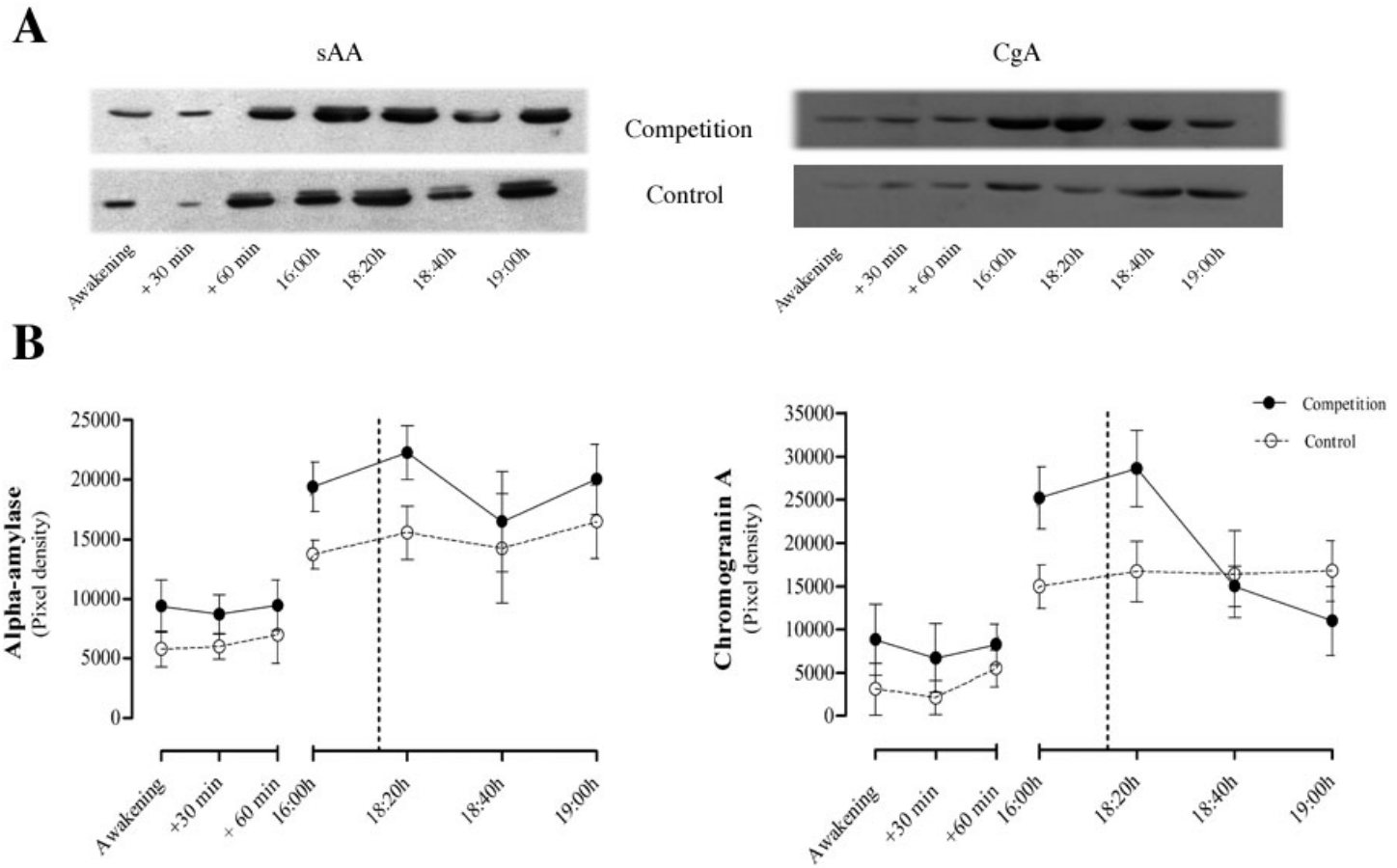
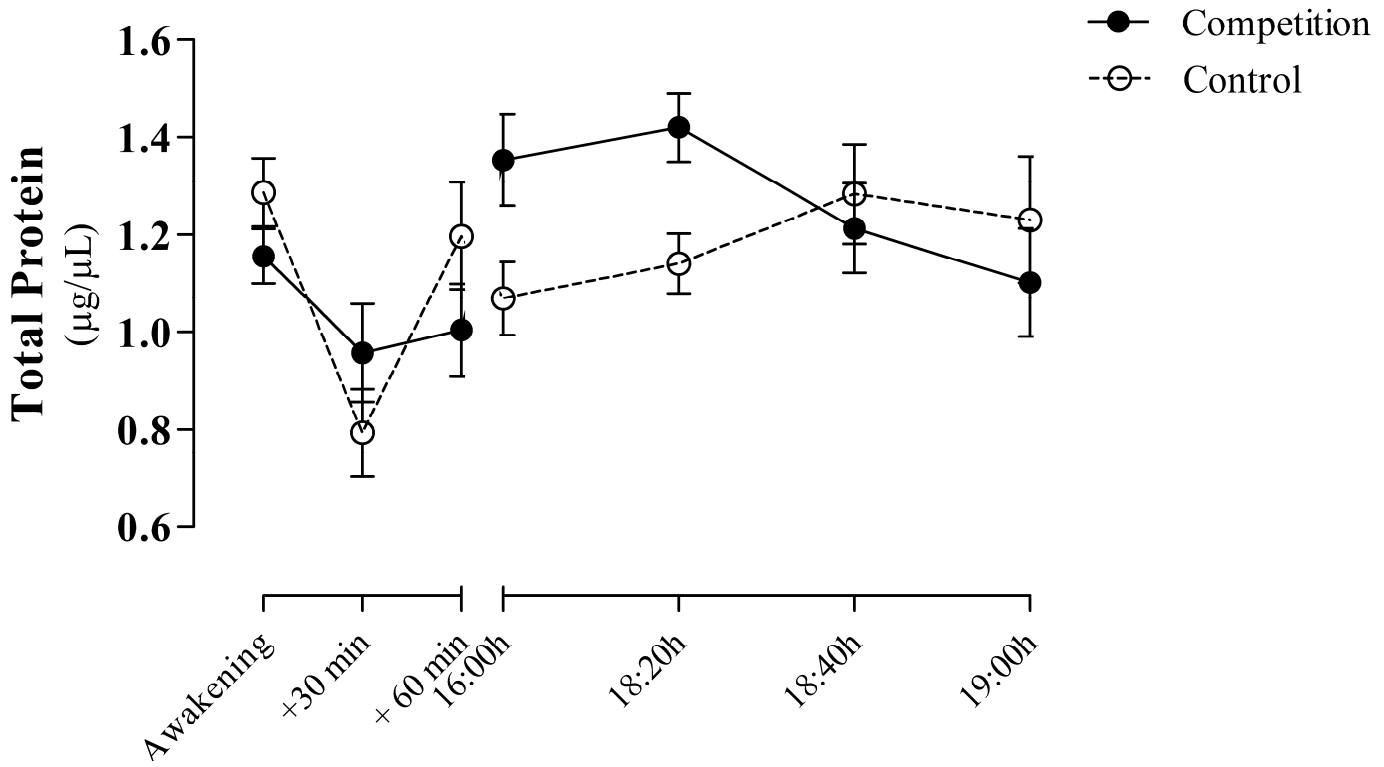


Figure 2. Diurnal Profile of the Concentration of Salivary Total Protein during Days of Competition and Control. The dotted vertical line indicates time of swimming. Values are means and error bars indicate SD.



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