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Androgens and dominance: Sex-specific patterns in a highly social fish (*Neolamprologus pulcher*)

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ABSTRACT

In most vertebrates, aggression and dominance are tightly linked to circulating testosterone. Fish, however, have two androgens (testosterone, T and 11-ketotestosterone, 11KT) that influence aggression and dominance. To date, few studies have compared the relationship between androgen levels and the outcome of aggressive contests in both females and males of the same species. To investigate sex differences in androgens we staged size-matched, limited-resource (territory) contests with 14 female–female and 10 male–male pairs of the highly social cichlid *Neolamprologus pulcher*. We then examined androgen levels in recently established dominants, who won the contest and subsequently acquired a territory (for 3 h), and subordinates, who lost and did not acquire a territory. Newly dominant females had higher plasma T but similar 11KT levels to newly subordinate females. In contrast, newly dominant males had higher 11KT but similar T levels to subordinate males. The ratio of 11KT to T, which demonstrates physiological importance of T conversion to 11KT, was positively correlated with submissive behavior in female winners, and correlated weakly with aggressive behavior in male winners ($p = 0.05$). These findings provide support for the hypothesis that different androgens play equivalent roles in female versus male dominance establishment, and suggest that relative levels of 11KT and T are implicated in female dominance behavior and perhaps behavior of both sexes.

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1. Introduction

Androgen hormones have been linked to aggressive behavior across a broad spectrum of vertebrates (reviewed by Nelson (2000)). Manipulation experiments have demonstrated that androgen removal decreases aggression while androgen treatment rescues or increases aggression (Edwards, 1970; Arnold, 1975; Balthazart, 1983; Moore, 1988; Kindler et al., 1991; Francis et al., 1992; Hume and Wynne-Edwards, 2005). These observations, paired with repeated demonstration that individuals with elevated androgen levels exhibit high levels of aggression (Lincoln et al., 1972; Scott et al., 1980; Johnsen, 1998) have led scientists to accept circulating androgens as potent mediators of male aggressive behavior. More recent findings have shown that female aggression also varies with circulating androgen levels (reviewed in Bouissou (1983); Staub and De Beer (1997)). Both male and female aggression and androgen levels have been strongly associated with dominance over conspecifics (Bouissou, 1983; Schoech et al., 1991; Desjardins et al., 2008b). To our knowledge, however, no study has measured androgen levels corresponding with dominance

establishment in both males and females, and sex-specific roles of androgens in dominance establishment remain poorly understood.

Neolamprologus pulcher, a group-living African cichlid, is an ideal model to test predictions on aggression and dominance establishment in both males and females. Highly territorial, this fish lives in groups composed of a dominant breeding pair and up to 20 subordinate helpers (Balshine et al., 2001; Heg et al., 2005). All individuals constantly reinforce their position within the group dominance hierarchy through a variety of aggressive, submissive and affiliative behaviors. As in all group-living species, predation events and natural deaths create vacancies in the dominance hierarchy. When these vacancies occur in top breeding positions, conspecifics from within or outside the group compete over the newly vacant position (Balshine-Earn et al., 1998; Stiver et al., 2006; Fitzpatrick et al., 2008). The victor of these competitions fills the dominant breeding position. Maintaining a position of dominance requires the strength and stamina for territory defense, subordinate policing and reproduction. These behaviors have been associated with circulating androgen levels in fish (Neat et al., 1998; Parikh et al., 2006), and androgen levels have been implicated in aggressive behavior during male dominance establishment across vertebrate

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taxa (Bouissou, 1983; Wingfield, 1985; Moore, 1988; Oliveria, 2004). Importantly, a previous experiment with *N. pulcher* found that winners and losers had equally elevated androgen levels immediately following a contest, although no changes in estradiol were seen in either sex (Desjardins et al., 2006). The androgen increases observed in both contestants may reflect short-term aggression-induced fluctuations rather than long-term patterns associated with status change, as established dominants in the field have higher plasma androgen levels than subordinates (Desjardins et al., 2008b).

The first aim of this experiment was to determine whether individuals successfully acquiring a territory for 3 h after completion of competition would exhibit higher levels of circulating androgen hormones than individuals who were unsuccessful in territory acquisition. The second aim was to explore whether females and males differ in androgen response to territory acquisition and establishment of dominance. To investigate endocrine and behavioral correlates of territory acquisition, we staged competitions for a shelter-containing territory between size-matched individuals of the cichlid fish, *N. pulcher*. As this fish species is highly philopatric and completely reliant on shelter in its territory for survival and reproduction (Balshine et al., 2001), we staged contests for shelter and then compared plasma androgens, relative gonad investment, and measures of aggression between competition winners and losers. Plasma T and 11-KT were assayed, as these are considered the most active androgens in females and males, respectively (Borg, 1994). Specifically, we predicted to find higher circulating androgen levels in both female and male individuals that successfully gained control of a territory (winners) compared to individuals that did not (losers).

2. Materials and methods

2.1. Study animals and holding conditions

Territory competitions were performed between 24 September and 26 November 2004 using *N. pulcher* held at McMaster University in Hamilton, Ont., Canada. Fish used in this study were derived from a wild-caught stock of fish originating from the southern shore of Lake Tanganyika, received in 2001 and 2002. To mimic natural lake conditions water temperature was kept between 25 and 28 °C and a 13:11 light:dark cycle was maintained. Fish were fed flake cichlid food daily, ad libitum. Tanks contained 2.0 cm of fine coral sand as substrate, an AquaClear Mini mechanical filter and a thermometer. Twenty male and 28 female subordinates were selected from stable 190-l group tanks (92 × 41 × 50 cm) for this study. Subordinate status was determined by repeated observations of submissive behaviors by the focal fish toward a dominant individual of the same sex. Fish were measured and given a unique fin clip for individual identification. The fish used had a mean standard length (\pm standard error) of 5.92 \pm 0.11 cm (range 4.50–7.98 cm) and mean body mass of 5.76 \pm 0.34 g (range 2.316–13.697 g).

Individuals were grouped into sex- and size-matched pairs, and then each member of a pair was randomly placed into one end compartment of a 30-l experimental tank (48 × 26 × 30 cm) partitioned into three equal 16 × 26 × 30 cm compartments. Each compartment was separated by one opaque and one transparent barrier. The central compartment (not containing any fish) contained a flowerpot half that served as a shelter and as the resource over which the animals competed. Paired, sex-matched fish always originated from different tanks to avoid effects of familiarity and previous interactions, and each fish was only used once. The mean size difference (\pm one standard deviation) between paired individuals was 0.20 \pm 0.03 cm (0.19 \pm 0.05 cm was the mean difference

between paired females and 0.22 \pm 0.04 cm was the mean difference between paired males).

2.2. Territory competition

Fish were placed in pairs in the experimental tank compartments. Tanks were completely covered with opaque plastic sheeting to occlude all light, and fish were left in the dark for 60 min to acclimate to their new environment. After 60 min, the plastic sheeting and opaque barriers were removed. The remaining transparent barriers allowed both fish to see each other and the shelter in the middle enclosure. This stage lasted for 10 min. Following this light acclimation period, transparent tank partitions were removed so that both fish were able to swim freely throughout the entire tank. All interactions were videotaped and aggressive behaviors were later scored from the videotapes by observers blind to the identities of the fish. Trials lasted for 20 min and were always conducted between 9:00 and 13:00 to control for daily variation in androgens and behaviors (Oliveira et al., 2001; Werner et al., 2003). All aggressive and submissive interactions were recorded (see Table 1 for an updated ethogram for this species). Time spent within the shelter was also recorded. At the end of each 20 min competition, winner status was assigned to the fish guarding the shelter and loser status was assigned to the fish furthest away from the shelter, often this individual was near the top of tank. One transparent partition was then replaced to sequester the loser into a compartment at one end of the tank (as before the competition). The winner retained access to the other two-thirds of the tank including the shelter. The two fish remained in this arrangement for 3 h, after which blood samples were collected. Studies have shown that both winners and losers can have similarly elevated androgen levels immediately after an aggressive interaction (Neat and Mayer, 1999; Desjardins et al., 2006); by waiting 3 h our samples reflected longer-term hormone patterns following social status establishment and territory acquisition.

2.3. Hormone assays

Blood collection and hormone assay protocols in this study were based on Desjardins et al., 2006, 2008b). Three hours after the competition was completed, both fish were caught, weighed, measured (standard length and total length), and anesthetized with a lethal dose of benzocaine (ethyl-*p*-aminobenzoate, 1.0 μ g/ml). Blood was collected by caudal severance with heparinized microcapillary tubes. The time from researchers approaching the tank after the experiment to completion of blood sampling was less than 3 min. Blood was spun at 8000 rpm for 10 min after which separated plasma was removed and frozen at -20°C for later measurement of T and 11-KT. A mean of 29 \pm 12 μ l of plasma was obtained from blood samples. Gonads, livers, and brains were removed and weighed. Fish were sexed by examination of gonads.

Steroid extraction from plasma was performed by shaking aliquots with 5 ml of diethyl ether, twice. The aqueous phase was frozen in a bath of acetone and dry ice, and the organic phase was decanted and dried. Dry extracts were resuspended in assay buffer and frozen at -20°C . Extraction losses were independently monitored by extracting five samples hot spiked with a known volume of radioactivity, and recoveries after performing a double extraction were 88 \pm 4%. Antibodies originated from Cayman Chemical. T and 11KT were measured by radioimmunoassay according to plasma steroid measurement techniques described by McMaster et al. (1995). Stated values are mean concentrations of duplicate aliquots whose values were within the 5–95% binding limits of the appropriate standard curve after linearization with a log–logit

Table 1
Ethogram outlining the aggressive and submissive behavioral repertoire of *Neolamprologus pulcher*.

Submission	Submissive posture	Focal fish directs head upward and tail downward
	Submissive display	Focal fish is positioned with a submissive posture accompanied by a quivering tail (or entire body quiver)
	Hook display	Focal fish swims toward another fish, bites or rams it, then turns away and performs a submissive display
	Flee	Focal fish quickly swims away from another fish
Non-contact aggression	Aggressive posture	Focal fish directs head downward and toward another fish
	Displace	Focal fish swims toward another fish, forcing it to move
	Puffed throat	Focal fish flares out its operculum and lower jaw cavity
	Head shake	Focal fish repeatedly thrashes its head from side to side
	Chase	Focal fish pursues another fish
	Pseudo-mouthfight	Back-and-forth movement occurs between two fish (as if about to mouthfight) but no contact made
Contact aggression	Ram	Focal fish makes contact with another fish using the head or mouth region, but jaws remain closed
	Bite	Focal fish bites another fish
	Mouthfight	Focal fish and another fish lockjaws and push against one another

transformation. Intra-assay coefficients of variation range from 4.8–7.8% for T and 5.1–9.2% for 11-KT. Inter-assay coefficients of variation did not exceed 13% in assays for T or 11-KT. All samples were run in a single assay. Assays for T and 11-KT were validated for this species prior to the experiment and are reported in Desjardins et al. (2006).

2.4. Ethical note

Behavior was closely and constantly monitored throughout the trials. It was determined *a priori* that a trial would be stopped if aggressive contact was maintained for over 30 s or any signs of injury were noted. No trials needed to be stopped prematurely. The protocols for catching, marking, and sampling the blood of fish were assessed and approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol #061059), and conformed to the guidelines prescribed by the Canadian Council for Animal Care.

2.5. Statistical analysis

Statistical analyses were performed using the JMP 5.1.1 statistical software package (version 5.1, SAS Institute Inc., 2004) on an Apple computer. Data were tested for normality using the Shapiro–Wilkinson test, transformed when necessary and analyzed with parametric tests when possible. Two-tailed tests were used throughout, and paired tests were used when comparing measures in paired fish. To maintain consistency with existing literature, gonadosomatic index (GSI) was used to measure relative gonad investment (calculated as *gonad mass/body mass*). A critical analysis of the use of GSI (see Tomkins and Simmons, 2002) suggests that other measures of relative gonadal investment may be superior, however our results did not differ regardless of which measure was used.

Some individuals had too small a blood volume to yield detectable hormone levels; these were deleted from the dataset. When the blood sample was large enough to reveal a hormone level but a non-detectable hormone level was nonetheless observed we estimated the minimum detectable level from the standard curve for this individual to ensure that the means were not artificially inflated by normal data. Inconsistent sample sizes are due to insufficient plasma in some samples to run one or both hormone assays.

Measures of total aggression and submission were calculated by summing the total number of aggressive and submissive acts, respectively. Aggressive behaviors were further categorized into non-contact and contact aggressive behaviors (see Table 1). To test for sex and status (winner, loser) differences in aggressive behavior we performed Wilcoxon signed-rank or Mann–Whitney U tests.

Sex and status differences in hormone levels were tested using two-tailed 2×2 factor ANOVAs, followed by post hoc *t*-tests to examine differences between paired fish. Tukey–Kramer corrections were used to correct significance for multiple comparisons. To test for relationships among androgens and relative gonad investment we used Pearson correlations. Spearman rank correlations were used for behavioral data, which could not be normalized.

3. Results

3.1. Do males and females have similar androgen responses to dominance establishment?

Female individuals that won territory competitions had higher levels of circulating plasma T than those that lost (paired *t*-test: $t_7 = -2.46$, $p < 0.05$; Fig. 1a), but there were no male winner–loser differences in plasma T levels ($t_8 = -1.13$, $p = 0.29$; Fig. 1a).

Females winners and losers did not differ in plasma 11KT levels ($t_8 = -1.65$, $p = 0.14$; Fig. 1b). Male winners, however, did have higher plasma 11KT than male losers ($t_6 = -2.90$, $p < 0.05$; Fig. 1b).

The plasma 11KT/T ratio, which indicates the physiological rate at which T is converted to 11KT (Oliveira, 2004), did not show winner–loser differences in female ($t_7 = -1.59$, $p = 0.16$; Fig. 1c) or male ($t_5 = 0.48$, $p = 0.65$; Fig. 1c) pairs.

3.2. Does gonadal investment relate to androgen levels or predict dominance?

No significant correlations were found between plasma androgens and relative gonad investment (all *p* values > 0.16). Relative gonad investment did not differ between female winners and losers (paired *t*-test, $t = -1.44$, $df = 9$, $p = 0.18$) or male winners and losers ($t = -1.16$, $df = 6$, $p = 0.29$).

3.3. Were fish that established dominance more aggressive during the contest?

Winners exhibited more aggressive behaviors (Wilcoxon signed-rank test, $Z = 4.54$, $p < 0.0001$), fewer acts of submission ($Z = -4.17$, $p < 0.0001$), and spent more time in the shelter ($Z = 4.42$, $p < 0.0001$) than losers. These patterns were maintained when we looked at female pairs only; female winners showed more aggression ($Z = 4.46$, $p < 0.0001$), less submission ($Z = -3.24$, $p < 0.05$), and more time in the shelter ($Z = 3.70$, $p < 0.001$) compared to female losers. Male winners were similarly more aggressive ($Z = 2.03$, $p < 0.05$) and less submissive ($Z = -2.04$, $p < 0.05$), but did not spend significantly more time in the shelter ($Z = 1.77$, $p = 0.08$) than male losers. Median, maximum, and minimum num-

bers of aggressive behaviors in female and male winners and losers are presented in Table 2. No sex differences were found in total number of aggressive behaviors (Mann–Whitney test, $U = 1.06$, $p = 0.28$), submissive behaviors ($U = -0.01$, $p = 0.99$), or time spent in the shelter ($U = 0.25$, $p = 0.80$). However, males performed proportionally more non-contact aggression relative to contact aggression compared to females (median proportions of non-contact aggressive acts in females and males were 78% and 100% of all aggressive acts, respectively, $U = 2.12$, $p < 0.05$; see Table 1 for ethogram).

3.4. Did androgens relate to aggressive or submissive behaviors?

Aggressive behaviors were not related to T or 11KT in any fish (all p values > 0.19). The 11KT/T ratio, however, had a near-significant positive correlation with the number of aggressive behaviors in male winners but not losers (Spearman's rank correlation, male winners $r_s = 0.95$, $p = 0.05$, male losers $r_s = -0.67$, $p = 0.15$). Similarly, the total number of submissive behaviors was not related to T or 11KT (p values > 0.19) but was positively correlated to the 11KT/T ratio in female winners ($r_s = 0.69$, $p = 0.04$). This ratio was not correlated with aggression in female losers, male winners or male losers (p values > 0.27).

4. Discussion

Androgen secretion patterns observed in female and male *N. pulcher* were in line with previous studies in this and other fish species; circulating T is higher in female fish while 11KT or the 11KT/T ratio is higher in males (Oliveira, 2004; Desjardins et al., 2008b; reviewed in Borg, 1994). Importantly, this is the first study to show that females and males of the same species have distinct hormone profiles associated with recent change in social status. Dominant females had higher T (but not 11KT) than subordinate females, while dominant males had higher 11KT (but not T) than subordinate males. Although pre-contest hormone levels were not measured (due to small amounts of blood in each fish), it is unlikely that these observed winner–loser differences were short-term effects of agonistic interactions. Sampling immediately following a contest revealed similarly elevated androgen levels in both winners and losers (Desjardins et al., 2006). The presence of a hormone difference 3 h after confrontation indicates that the appearance of physiological winner–loser effects requires a longer time scale than immediate aggression-induced androgen elevation. Winner–loser effects in some fish have been shown to last 48 h (Hsu and Wolf, 1999), and have been suggested to be mediated by androgens. This concurs with previous findings that higher plasma androgens are not a predictor, but instead a result of hierarchy formation (Oliveria et al., 1996). Our observation that newly dominant and subordinate fish show corresponding differences in androgens well after agonistic behavior ends indicates that these different androgens may mediate lasting effects of dominance in females and males.

Gonads, as the primary source of plasma androgens in fish, could be expected to have different effects on circulating androgen levels, depending on (gonad) mass. In established groups, dominant individuals have higher androgen levels and greater relative gonad investment than subordinates (Fitzpatrick et al., 2006; Desjardins et al., 2008b). In this study, plasma androgens did not correlate with relative gonad investment in any class of fish (female or male winners or losers), nor did relative gonad investment predict winners and losers. Size matching of the two subordinates and the short-term nature of the study likely precluded any relationship emerging between gonadal development and androgen levels or social status.

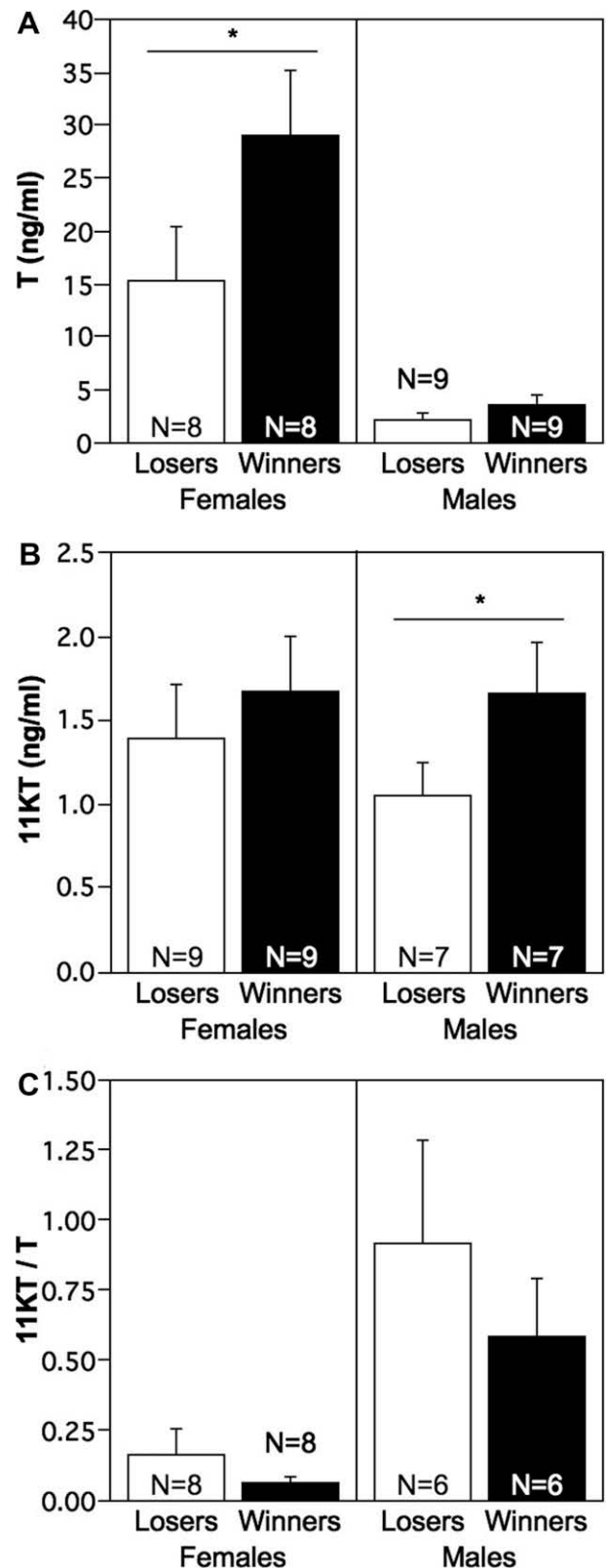


Fig. 1. Mean (\pm standard error) of plasma (A) T, (B) 11KT, and (C) 11KT/T ratio levels in female and male winners and losers after 20-min male–male or female–female resource competitions. Asterisks indicate a statistical difference at $\alpha = 0.05$ with a two-tailed t -test, paired for within-trial (within-sex) comparisons, unpaired for between-trial (between sex) comparisons. Plasma collected from some individuals was insufficient to perform one or both hormone assays, thus pairwise comparison could not be performed and samples sizes are less than the total number of pairs tested.

The fact that winners performed more aggressive acts and losers more submissive acts supports the fact that these behaviors are important signals by which social rank is determined. Simply put, more aggressive fish won contests (control of the resource). Interestingly, although males are often considered more aggressive, no sex difference in total aggression was observed. Both female and male dominants are strongly territorial and aggressive in this species (Desjardins et al., 2008a), but females often show more aggression (Balshine et al., 2001; Aubin-Horth et al., 2007). Of the total aggression displayed, a greater proportion of the male behavior was non-contact displays rather than overt contact behaviors, while females tended to perform more risky (Hurd and Enquist, 2001) contact aggressive behaviors, indicating that females are willing to take greater risks to establish or defend a territory. In *N. pulcher*, dominant females control only a single territory while dominant males may often control several territories (Desjardins et al., 2008a). Hence the loss of a single territory would incur a greater cost to a dominant female compared to a dominant male who might have other reproductive options. Males in this study were less willing to engage in riskier contact aggression.

Interestingly, the 11KT/T ratio tended to be positively correlated with the degree of aggressive behavior in male winners, although this result was non-significant ($p = 0.05$). Agonistic behavior in winners might increase the conversion of T to 11KT just enough such that a relationship is only seen with this ratio and not with either androgen alone. Conversely, in female winners the 11KT/T ratio was positively correlated with submissive behavior. Gonadal hormones have been implicated in submissive behavior in female rodents (Faruzzi et al., 2005), and non-gonadal steroids have been shown to be related to submissive behavior in another cichlid fish species (Munro and Pitcher, 1985). More work is needed to resolve how relative levels of androgens may play a permissive role in social behavior across vertebrates.

This study is the first to compare androgen responses to dominance establishment in females and males of the same fish species under identical conditions. Additionally, this is also the first demonstration that two different androgens, T and 11KT, appear to play the same role in dominance establishment of females and males, respectively. These findings suggest that behavioral and physiological changes resulting from ascension to dominant status are potentially mediated by different androgens. The physiological functions of these respective androgens in adult females and males remain poorly understood, however, and more research is needed to resolve the roles of androgens in changes that occur with a rise in status. In addition, while a clear picture is forming on how androgens modulate behavior at the level of a dyadic resource contest, the applicability to establishment of social rank in large, complex groups remains unknown.

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Table 2

The median number (and range) of aggressive and submissive behaviors performed by female and male losers and winners during 20 min contests. See Table 1 for a list of all aggressive and submissive behaviors.

	Females		Males	
	Losers	Winners	Losers	Winners
Total submission	6.5 (0–19)	0 (0–7)	3 (0–25)	0 (0–9)
Total aggression	1 (0–27)	10 (3–57)	2 (0–47)	12 (4–50)
Non-contact Aggression	1 (0–18)	7 (0–38)	2 (0–45)	11 (4–43)
Contact aggression	0 (0–9)	2.5 (0–25)	0 (0–2)	0 (0–17)

with respect to the statistical analyses. We also thank anonymous referees for comments on the paper. This work was supported by a NSERC Discovery Grant awarded to S.B. and an NSERC doctoral PGS award to J.K.D.

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