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Maternal care and altered social phenotype in a recently collected stock of *Astatotilapia burtoni* cichlid fish

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Synopsis For over 30 years, the African cichlid fish, *Astatotilapia burtoni*, has been an important model system for studying the mechanisms underlying socially mediated behavioral change, with the focus being the dominance behavior of males. A recently collected wild-stock (WS) of this species invigorates interest in parallel studies of females' behavior. Here, we describe a robust 'good-mother' phenotype, increased maternal affiliation in fry, and subtle differences in males' behavior that are exhibited by this new stock. While the females of both the laboratory-stock (LS) and the WS brood the developing fry in their buccal cavity, only the WS continues to provide maternal care after initial release of the fry while the LS engage in filial cannibalism. We show that weight loss during starvation, either during brooding or with restriction of food, is greater in the LS than in the WS; thus, the observed behavioral differences may be tied to metabolic differences. The WS also exhibits a robust androgen response to challenge during the maternal care phase. Given the increasing power of genomic tools available for this species, the comparison of these two stocks will offer the opportunity to investigate the genetic and genomic basis of behavioral differences.

Introduction

> . . . In front goes the male, dropping their milt which the females, following behind, gulp down. It is this that causes the females to conceive. . . [the females] do just what the males did . . . dropping their grains of spawn, a little here, a little there, while the males who follow behind swallow them up. Each of these grains of spawn is a fish embryo; some of them escape and are not swallowed up by the males, and it is these which afterward grow to maturity.
>
> Herodotus *The Histories* Book II (93)

As pointed out by scholars (Asheri et al. 2007), the true behavior, described here for Tilapia, has been 'badly misunderstood' in that Herodotus (2003) believes the fry are eaten rather than brooded, but one can hardly fault him for not correctly comprehending the complex and varied behaviors of cichlids. We continue to discover novel behavioral phenotypes even within the African cichlid species that, perhaps, is studied the most in this regard, *Astatotilapia burtoni* (formerly *Haplochromis burtoni*). This species, from Lake Tanganyika and its tributary rivers, provides a model for social regulation of behavior that has been pursued at a behavioral, physiological, and molecular level (e.g. Fernald 2004; Burmeister 2007; Renn et al. 2008). Appropriately, research with the *A. burtoni* model species stands to benefit from the genome-sequencing project to be undertaken at the Broad Institute, funded through NHGRI (Kocher et al. 2006). While cichlids in general provide a model system for studying the evolution of adaptive phenotypes, this species in particular has been a fruitful model system for the study of phenotypic plasticity and social dominance for over 30 years. The modern techniques, novel approaches, and new ideas applied to *A. burtoni* continue to broaden our understanding of the biological basis of behavior and usher in a new model organism. In combination with other emerging models for social behavior, a theoretical model for sociogenomics has been developed allowing an investigation of interactions between genes and environment on multiple timescales, from rapid physiological change, through ontogenetic plasticity, including epigenetic regulation and even incorporating evolutionary adaptation (Pollen and Hofmann 2008; Robinson et al. 2008).

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A. burtoni males have been a long-standing model for behavioral plasticity

Fish species in general have emerged as important models for studies of social behavior and its biological underpinnings. The African cichlid fish, *A. burtoni*, in particular, has become an important model system for studying the mechanisms underlying socially mediated behavioral change. In this species 20–30% of males are dominant, slow growing, brightly colored (either blue or yellow), and actively defend territories for mating. The remaining subordinate males mimic females by schooling and displaying cryptic coloration (silver gray), while experiencing faster growth (Fernald 1977; Fernald and Hirata 1977a, b; Hofmann et al. 1999). The associated behavioral and physiological characters are highly plastic and are influenced by the past (Fernald 1980) and immediate social environment (Fernald and Hirata 1977b). In both the laboratory and the field, several times during its life, an individual male switches between the dominant and subordinate phenotypes depending upon his ability to obtain and maintain access to a territory through aggressive encounters with other males.

In the laboratory, *A. burtoni* has been the focus of hormonal and molecular studies related to a broad range of phenotypic traits that are effected by the plastic switch (for reviews, see: Fernald 2002; Hofmann 2003; Burmeister 2007). This complex, reversible, and repeatable switch is orchestrated through variation in many components. Neuroendocrine pathways regulating growth (Hofmann et al. 1999), androgen production (Parikh et al. 2006a, b), stress response (Fox et al. 1997), and neurophysiology (Greenwood and Fernald 2004) change in a complex fashion as animals undergo phenotypic change (Fig. 1). For example, expression and release of neuropeptides and neuromodulators such as gonadotropin releasing hormone (GnRH1) (White et al. 2002) and somatostatin (Hofmann and Fernald 2000; Trainor and Hofmann 2006) are higher in dominant males. Neuroanatomical studies have localized both long-term (GnRH1: White et al. 2002) and short-term (egr-1: Burmeister et al. 2005) changes in gene expression to hypothalamic nuclei and the pituitary (GnRH-receptors: Au et al. 2006; steroid receptors: Burmeister et al. 2007).

Most recently, this cichlid species emerged not only as a physiological and behavioral model for social plasticity, but also as a genomic model (Hofmann 2003; Robinson et al. 2008). Gene expression profiling, applied to this system, has identified gene modules that underlie the complex socially regulated switch (Renn et al. 2008, Larkins-Ford et al. in preparation). Genes coding for structural proteins such as tubulin and actin, proteins that bind scaffold elements, such as septin 7 and ELF-1a, as well as axon-growth-specific genes, neuroserpin and neuromodulin, were all up-regulated in territorial males, strongly suggesting axonal growth, reminiscent of the remodeling of neuronal architecture for the brain nuclei seen in seasonal accession to territoriality and mating that is accompanied by increased testosterone levels in song birds (reviewed in Arnold 1992). As one might expect, many neuroendocrine and neurotransmitter genes were regulated according to social status. GnRH1, AVT galanin, somatotropin, prolactin, somatolactin proopiomelanocortin, and a GABA-A receptor were

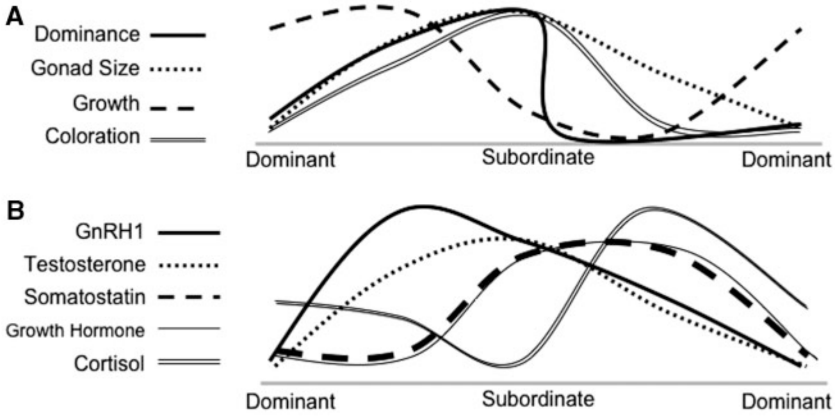

Figure 1 Schematic representation of (**A**) phenotypic and (**B**) physiological characteristics associated with dominant and subordinate male phenotypes in *A. burtoni*. The graphs are based on the following studies: gonad size (Hofmann and Fernald 2000), growth (Hofmann et al. 1999), GnRH1 (White et al. 2002), testosterone (Francis et al. 1993; Parikh et al. 2006a, 2006b), somatostatin (Hofmann and Fernald 2000), growth hormone (Hofmann et al 1999), cortisol (Fox et al. 1997). The waveforms are only approximations and details are dependent upon an animal's past social rank experience (Figure taken from Renn et al. 2008).

found to be up-regulated in dominant males, whereas a homolog of cholecystokinin and natriuretic peptide, and at least two subunits of the kainate-type glutamate receptor were found to be up-regulated in subordinate males (Renn et al. 2008). Contrary to naive expectation, using a ‘module’ approach to gene expression analysis, it was found that dominant males are not simply ‘super males’ (i.e. a phenotype that over expresses male-specific genes), but instead, many of the genes up-regulated in females are also important for determining social status in males. This ‘systems level approach’, which considers gene regulation in modules and patterns, complements the gene-list approach and allows the discovery that co-regulated gene sets are associated with social status. In combination with current cichlid genome projects, these results pave the way for future genomic studies to uncover how molecular modules are associated with behavioral or physiological measures of social status in different contexts and also to address the molecular mechanisms by which social cues are transduced to lasting changes in phenotype (see Burmeister 2007).

Less is known about plasticity of behavioral phenotypes in female *A. burtoni*

Given the wealth of knowledge regarding phenotypic plasticity in *A. burtoni*, it is surprising that so little is known regarding behavioral changes in females. These females school in the wild, visiting the lekking males to select a mate with whom to spawn, after which they collect their eggs in their mouths and leave the male’s territory to brood the offspring. Although, initial studies in *A. burtoni* noted that ‘females with young may defend territories for short periods, and during this time may acquire head-coloration consisting of the eye-mouth bar and sometimes the two forehead stripes’ (Fernald 1977; see also Tacon et al. 1996 for tilapia), all available field data (Fernald and Hirata 1977a) and the majority of the laboratory-based studies in *A. burtoni* are focused on only males’ behavior and physiology as regulated by social interaction.

The regulation of females’ behavior and physiology is reported to be driven by ‘internal reproductive state’ because it was believed that ‘females have no apparent social hierarchy’ (White and Fernald 1993). Similarly, in another mouthbrooding cichlid, the Black Chinned Tilapia, while both testosterone and estradiol are elevated on the final days of brooding, and aggressive behavior is increased, the agonistic repertoire of these brooding females seems to lack the stereotyped displays exhibited by males, thus differentiating males’ territorial aggression from potential maternal aggression (Oliveira and Almada 1998). However, in neither of these studies were physiological measurements made when mature fry were present. With regard to behavior, the female’s affiliative preference for a male of social status is known to alternate between her gravid and non-gravid reproductive states (Clement et al. 2005). Her switch in behavioral preference has been correlated with levels of testosterone and estradiol in the blood and with GnRH1 expression (Martin 2004; Desjardins et al. in press) (Fig. 2). As such, the hormonal changes during the reproductive cycle have been characterized in correlation with gonadosomatic index (GSI) with little attention to behavior. When females release their fry, circulating plasma levels of 17β-estradiol and testosterone are low, the ovaries are regressed, and little GnRH transcript is produced in the brain (Martin 2004). Interestingly, testosterone and estradiol concentrations increase after release of the fry and are thought to peak just before spawning (Fig. 2). However, few behavioral data regarding social interactions are available for such studies (but see Clement et al. 2005), and furthermore, females were not allowed to interact with their fry as they were artificially stripped from the buccal cavity or removed upon release.

Similarly, almost no attention has been paid to social interactions and potential for dominance hierarchy among the schooling females (but see Desjardins et al. in press). It was previously noted that in the absence of males, a few individual females in an all-female population would take on male-like behaviors, display eye-bars and body pigment (though to a lesser degree than did males). This male-like transition was tested for the possibility of sex change, but no gonadal transition accompanies the behavioral switch (Rhodes 1995;

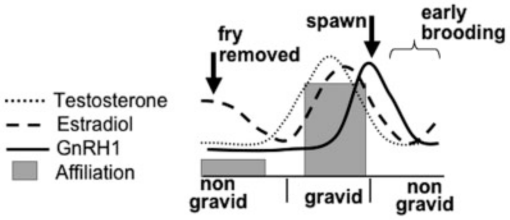

Figure 2 Schematic representation of hormone and behavior through female reproductive cycle in *A. burtoni*. The graphs are based on the following studies: Affiliation: Clement et al. 2005; Estradiol and Testosterone: Martin 2004; Desjardins et al. in press; GnRH1: White et al. 2002.

Renn personal observation). In these all-female populations, testosterone levels were significantly higher in the male-like females compared to non-aggressive females, and tended to be higher than those of brooding females, although all female testosterone titers were significantly lower than in either male phenotype (Fraser et al. in preparation). This artificially induced aggressive phenotype reveals that the behavior and physiology of females is sensitive to social environment, at least in an extreme sense. In summary, despite the wealth of information concerning the physiological basis and consequences of social interaction in males, few data exist in this emerging model species for natural behaviors of females.

There are behavioral differences among *A. burtoni* stocks

The majority of research described above for *A. burtoni* has been conducted with a single fish stock that was collected from a site at the north end of Lake Tanganyka in Burundi in 1977 (Fernald 1977). This stock has been maintained in captivity under research laboratory conditions for over 30 years. The stock is not propagated with any maternal care, but rather the fry are removed from the buccal cavity at approximately 14 days post-fertilization when the fry are able to survive independently. The laboratory-stock (LS) fish have been provided ample territories in the form of terra cotta pot shards and have been maintained in relatively high density. There has been little effort to avoid inbreeding and it is likely that substantial artificial selection has occurred.

In 2005, a new wild-stock (WS) was collected from the Kalambo River on the Tanzanian and Zambian border above the 235 m Kalambo falls near the village of Kalambo toward the southern end of Lake Tanganyika. This stock (~100 individuals) has not been influenced by years of artificial selection in a research environment. Therefore, this stock represents a valuable tool with which to address differences in the genome regulation and possibly the genetic variation that is responsible for the observed behavioral differences described below. A recent survey of cichlid primers for microsatellite loci, first identified in Tilapia (Lee et al. 2005) and also shown to exist in *M. zebra* from Lake Malawi (Alberston et al. 2003), identified 41 of the 46 tested primer sets that amplify product in *A. burtoni* (Kunkle et al. in review). While further work is required to identify those genetic loci that will be useful markers for differentiating WS and LS, these tools demonstrate the strength of the cichlid system for the generation and application of genomic tools that will be utilized to study the genetic and genomic basis of complex phenotypes from an ecologically and evolutionarily relevant approach. Here we provide an initial report of the behavioral differences between the well-studied LS and this recently collected WS of *A. burtoni*. In particular, observations of this stock cause us to rethink our assumption about the aggressive behavior of females and therefore the molecular mechanisms that underlie it (see below).

Re-evaluation of *A. burtoni* females: a model for maternal care and aggression

Maternal aggression has long been recognized as a unique behavior and the majority of studies of maternal behavior have been conducted in rats, which provide maternal care upon parturition by the licking, grooming, and retrieval of pups. The stimulus needed to both attend young and attack potential intruders has been suggested to represent an evolutionary trade-off, such that expression of the aggressive behavior can bring harm to the parent, but might dramatically increase the survival and fitness of the offspring (Lonstein and Gammie 2002). Termination of maternal behavior is expected when the physiological costs outweigh the benefits. If the survival of offspring without care is low, as is often the case in fish, it may be adaptive for the parent to cannibalize the brood, and thus recover some energy from the aborted breeding attempt. Theoretically, factors favoring cannibalism include small brood size, increased predation risk, increased availability of reproductively viable males, and nutritional needs of the mother (Manica 2002). In most species, parental care is the rule and cannibalism is an exception.

Because propagation of the LS has relied on stripping the fry from the buccal cavity and raising them in the absence of maternal care, this stock has not been subjected to ecological or selective pressure against filial cannibalism after the fry are released. However, the WS, now only one generation removed from the natural environment, is expected to show more natural parental behaviors. When we observe the WS housed in standard stock tanks, the population density is such that a female cannot defend her offspring. However, when we provide females with a territory that is protected by a clear plastic barrier, allowing visual and olfactory contact without a physical threat, differences in maternal care between stocks become apparent. Females of ~6 months of age (i.e. not likely the first brood) were selected for observation when they were 2–3 days post spawning. WS females and LS females do not differ significantly with regard to the number of days from spawning to release of fry (WS: 17 ± 1.59, LS: 18.75 ± 2.1). Similar to the LS, WS females have been seen to spawn 17–28 days after releasing their first brood if it is removed. This duration of the reproductive cycle is consistent with previous findings (Martin et al. 2003). However, both wild-caught females and the first-generation progeny of these wild-caught females are more likely to be successful in raising a brood 5 and 10 days after release than LS, while the majority of LS females engage in filial cannibalism under the same conditions (Table 1). These first-generation WS progeny had been raised according to standard laboratory procedures, being taken from the maternal buccal cavity at 10–14 days after fertilization and being raised in the absence of parental care. Therefore, the maternal behavior is probably not due to maternal affects unless they occur while the embryos develop. These results suggest that the observed 'good-mother phenotype' in the WS is due to underlying genetic variation between the two stocks.

Females in this new WS exhibit stereotyped aggressive behaviors directed toward males on the opposite side of the clear barrier or toward a smaller male intruder introduced into their own compartments. These lateral threats, border threats, and tail quivers appear similar to those studied in males. As noted in the original field study, these females defend a territory and protect fry against intruders. These behaviors appear to be part of a normal and strong maternal-care repertoire not previously studied in *A. burtoni*. The WS females continue to take the fry back into the buccal cavity periodically for up to 10 days after initial release, although there is considerable individual variation, possibly due to brood size. WS females take their fry into their mouths just before lights out for several nights after release. The fry appear to use the eye bar as a trigger to enter the mouth, and will even attempt to enter the mouth of a dominant male across a clear divider if the female is not present (data not shown). It is not known whether the timing of shelter seeking is due to circadian behavior of the fry or of the mother (for diurnal physiology in *A. burtoni*, see Halstenberg 2005); however, it is clear that this and other distinct examples of maternal care in WS females represent relevant social interactions that cannot be accurately studied in populations that are many generations removed from natural environmental conditions.

Table 1 Stocks differences in brood survival

	Broods released	Brood present			
		Day + 1	Day + 3	day + 5	day + 10
Wild caught	18	18	17	16	15
WS F1	9	9	5	8	8
LS	12	7	2	1	1

Values indicate the number of broods for which even a portion of the fry was not lost to maternal cannibalism on the corresponding day after release.

A. burtoni females as a model for feeding regulation

Mouthbrooding appears in at least eight families and 53 genera of teleost fish (Oppenheimer 1970), including several hundred cichlid species (Fryer and Iles 1972). While the fertilized eggs are in the buccal cavity, the mother mouths or cleans the eggs by sucking away loose particles, churning the young in her buccal cavity and moving the brood around in ways that enhance respiration by her young (Keenleyside 1991). Once the yolk sac is absorbed and the wrigglers mature to the fry stage, the mother releases the fry from her mouth and resumes her omnivorous feeding. Throughout the mouthbrooding period, females voluntarily starve themselves (Oppenheimer 1970; Smith and Wootton 1994). This new WS of *A. burtoni* offers a unique model in which to pursue the biological/physiological basis and consequences of this behavior due to the wealth of behavioral, neural, hormonal and now genomic work available for this species.

During this period of oral brooding, known as the obligatory period, maternal growth is stunted (Fryer and Iles 1972) and results in a wasting of body mass. The diminished growth in brooding females affects the reproductive rate as evidenced by an increased duration until respawning (Smith and Wootton 1994, 1995). 'Brood care motivation' (the ability of a female to keep the brood in her mouth without eating the fry) and hunger have been quantified in another mouthbrooding cichlid, *Pseudocrenilabrus multicolor*, from Lake Victoria (Mrowka 1984). These traits were found to differ according to experience such that first-time spawners maintained brood care motivation for several days when the brood was prematurely removed, whereas it disappeared within a few hours for non-first-time spawners. When offered regular feed, the females that lacked brood care motivation ate as much as non-brooding control females, a result that was interpreted as similar levels of hunger. Meanwhile, females with high levels of brood care motivation ate half as much as non-brooding control females, which was interpreted as reduced hunger. This ontogenetic plasticity in behavior can serve as a model for feeding regulation and disorders in humans. Furthermore, potential differences in LS and WS offer the opportunity to address the genetic contribution to variation in feeding regulation.

This inhibition of food consumption in mouthbrooding females is thought to be controlled by two mechanisms: the short-term physical stimulus of eggs in the buccal cavity and the long-term neural mechanisms that regulate feeding. These short-term and long-term mechanisms of inhibition of feeding during mouthbrooding may be related to the short-term and long-term mechanisms for regulation of food intake that are well studied in mammals. In mammals, short-term feeding regulation again involves a physical stimulus: the distension of the esophagus, stomach, and intestine due to the presence of food (Konturek et al. 2004). Meanwhile, the long-term regulation of food intake involves a complex integration of central neuropeptides and peripheral signals affecting the brain in ways that maintain homeostasis or energy balance throughout the body. Importantly, this complex integrative mechanism appears to be conserved across vertebrates such that many neuropeptides and hormones, such as neuropeptide Y (Sundstrom et al. 2008), agouti-related protein (Song et al. 2003), pro-opiomelanocortin (Hansen et al. 2003), α-melanocyte-stimulating hormone (Forlano and Cone 2007), and their respective receptors (Logan et al. 2002; Ringholm et al. 2002; Metz et al. 2006; Larsson et al. 2005) are well conserved even in fish (Lin et al. 1999; Volkoff et al. 2005). The exploration of differences in the ontogenetic plasticity in maternal feeding behaviors between both stocks may provide insight into molecular differences in the regulation of feeding in humans' feeding disorders, such as obesity and anorexia.

To compare the differences in metabolic response, we established three experimental groups for each stock. The 'brooding' group included females that had spawned within 2 days, while the starved and fed groups included non-brooding females without regard for date of previous spawning. All animals were housed individually in 1.5-liter tanks with continual circulation and filtration. The fed group was fed flake food once daily *ad libitum*, the brooding group was fed minimal flake food to provide stimulus to feed, and the starved group were given no food at all. The wet body weight and the standard length were measured to the nearest 0.01 g and 1 mm at the beginning of the 10–14 day isolation. Any female in the starved or fed condition that spawned in isolation was eliminated from the study. All treated fish were weighed, measured, and sacrificed after 10–14 days of isolation (IACUC# 103.2007). Ovaries were fixed in 4% paraformaldehyde PBS, pH 7.7, and brain tissue was stored in RNA-Later (Ambion) for future studies of gene expression. Gonad somatic index (GSI) was calculated as the ovary mass divided by the difference between body and ovary weight, multiplied by 100. During the dissection, the presence or absence of food in the intestine and stomach of the fish was noted. None of the starved or brooding fish had any food in their intestines or stomach. One of the eight fed females had no food in her stomach during the dissection. All of the starved and brooding females, but only three out of eight fed females, had hypertrophic green gall bladders indicating retention of bile. Several studies have documented changes in gall bladder function for fish that are starved (e.g. McCormick and Podoliak 1984) or socially stressed (Earley et al. 2004). Either or both of these factors may influence gall bladder function in brooding females.

As expected, starved fish and brooding fish tended to lose body mass and fed fish gained mass throughout the experiment (Fig. 3). Fed animals also tended to have a higher GSI than either starved or brooding animals, although no between-stock difference was found in this measure of ovarian condition. Fed LS females gained a greater percentage of body mass than did WS females (LS: 15.3% ± 6.30; WS: 2.42% ± 4.28; *t*-test, $P = 0.0240$). Similarly, starved LS females lost a greater percentage of their body mass than did WS females (LS: 21.3% ± 3.89; WS: 8.60% ± 2.26; *t*-test: $P = 0.0262$). While the same trend held for brooding females, the difference was not statistically significant (LS: 15. 2% ± 4.80; WS: 6.20% ± 0.57; *t*-test: $P = 0.1081$). These results suggest that LS females have an increased sensitivity to food availability. They tend to grow more quickly when food is available and lose body mass more quickly when food is not available. This suggests a reduced ability to regulate metabolism that may be the result of relaxed selection in the laboratory environment. This increased loss of body mass during brooding could contribute to the observed 'bad-mother' phenotype seen in the LS females.

A. burtoni females as a test of the challenge hypothesis

The challenge hypothesis, originally put forth by Wingfield et al. (1990), has since become central to

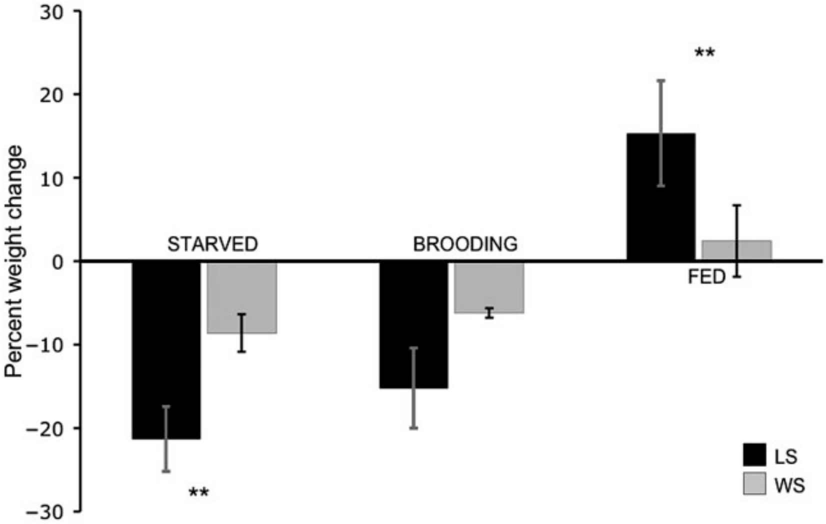

Figure 3 Stock differences in body weight change. The average percent weight change in LS (black) and WS (gray) females under starved, brooding, and feeding conditions (n = 4 each) for 10–14 days in response to feeding conditions. Error bars denote standard error. Asterisks indicate $P \leq 0.05$

understanding males' aggressive behavior through the lens of endocrinology as it relates to the ecology of a species. This theory posits an increase in androgens as a result of a territorial intrusion, especially in monogamous species or those that exhibit a high level of parental investment. In contrast, those species that must maintain territories through repeated agonistic encounters, often polygynous species, or those with low parental investment, might see an increase in aggression without a corresponding spike in androgens because hormone levels in males of these species are already maintained at maximal levels. Detrimental consequences of high androgen levels, in addition to the postulated consequences for paternal care, are thought to set baseline levels (Wingfield et al. 2001).

While originally proposed to explain conflicting results from studies in birds as a consequence of mating system, this hypothesis has more recently been tested in mammals (e.g. Ostner et al. 2002; Archer 2006), insects (e.g. Trumbo 2007), lizards (e.g. Moore 1987) and fish (e.g. Hirsenhauser et al. 2004) with more or less consistent results (for reviews, see: Hirsenhauser and Oliveira 2006; Goymann et al. 2007; Moore 2007). These studies have primarily focused on androgen response in males, addressing trade-offs between aggression and paternal behavior, often in the form of time–budget restrictions between parental investment and competition with other males. Despite the wealth of information for applying the challenge hypothesis to males, only a few studies have investigated hormone response to agonistic encounters in females. Female birds appear to respond with an increase in testosterone (e.g. *Prunella modularis*: Langmore et al. 2002; *Thryothorus leucotis*: Gill et al. 2007). Similarly, female fish showed an increase in both testosterone and 11-ketotestosterone (an important fish androgen) (Desjardins et al. 2006). However, female mice (*Peromyscus californicus*) showed a reduction in progesterone, suggesting an inhibitory effect of the hormone on aggressive behavior (Davis and Marler 2003), and the song sparrow (*Melospiza melodia*) showed no hormonal response despite behavioral response to intrusion into the territory (Elekonich and Wingfield 2000).

A recent study reports that aggression modulates androgens in female *A. burtoni* only when they are gravid (ready to spawn) but not during parental care at the early mouthbrooding stages (Desjardins et al. in press). It is supposed that female aggression, facilitated through androgen responsiveness, serves gravid females when they congregate near the lekking males. Taking advantage of the good-mother phenotype in the WS, we asked whether aggression modulates androgens at a later maternal stage when the fry are free swimming and the female would be defending a territory. Females were housed individually with no social contact throughout brooding. Control animals were sacrificed at 1:00 PM 4 days after initial release of the fry, and blood was collected by caudal severance following anesthesia with MS-222. Experimentally challenged females experienced a 10-min interaction at noon on day 4 after release. The intruder was a conspecific male ~50% her size in order to present a threat to the fry but

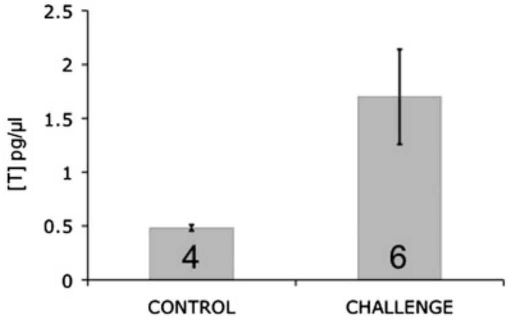

Figure 4 Testosterone concentration in plasma for control and challenged females as measured by ELISA. Error bars denote standard error, numbers denote sample size (*t*-test: $P=0.058$).

ensure the female's successful defense of the fry. Collection of blood occurred 1 h later at 1:00 pm. Plasma was separated by centrifugation and steroids were extracted with di-ethyl ether for ELISA measurement (Assay designs). We found a robust androgen response in the challenged females compared to controls (control: 0.483 ± 0.027 pg/µl; challenged: 1.70 ± 0.441 pg/µl; *t*-test: $P=0.058$) (Fig. 4). However, among the six females that experienced the territory challenge, there was no apparent correlation between the females' hormone level and the number of aggressive attacks directed toward the intruder (Pearson's correlation: $r=-0.44$, $P=0.38$). These data suggest that *A. burtoni* females maintain testosterone at subthreshold levels during the brooding period, supporting the implications of the challenge hypothesis that androgens are kept at low levels except in the case of a territorial challenge. Agonistic social stimulus by a conspecific appears to cause a hormonal response in females, thereby promoting maternal aggression. The fry themselves and their behavior may also provide unexplored social stimuli that influence variation in maternal behaviors.

Does the behavior of fry contribute to differences in maternal phenotype between stocks?

While some attention has been paid to neural development (Francis et al. 1994), development of the visual system (e.g. Fernald and Wright 1985), the ontogeny of behavior and coloration of males (Fernald and Hirata 1979), and early social regulation of growth (Fraley and Fernald 1982), the behavior in cichlid fry has also been studied in regard to kin recognition, using mouthbrooding cichlids as a model for social bond formation (e.g. Russock 1999). Kin recognition has been demonstrated to involve chemical recognition both by the parents (Myrberg 1975; Mackay and Barlow 1976) and the offspring (Barnett 1982, 1985), as well as visual cues (Russock 1986), being also dependent upon the age and behavior of fry (Noakes and Barlow 1973). More recently cross-fostering experiments between closely related species (e.g. heterospecific cross-fostering) have demonstrated that learning, in the form of sexual imprinting, may help to maintain reproductive isolation (Verzijden and ten Cate 2007). Young females from these cross-fostered broods develop a sexual preference for males of their foster mothers' species (Verzijden et al. 2008). Suprisingly, the social behavior of males is not altered by heterospecific cross-fostering. Both the mate choice and dominance patterns of males from these heterospecific cross-fostered broods retain conspecific mate preference and aggression (Verzijden et al. 2009). With *A. burtoni*, we conducted a preliminary study on stock cross-fostering to determine whether the early behavior of WS fry differ from that of the LS.

In order to initiate cross-fostering, two brooding females, one from each stock at 2–4 days post spawning, were gently forced to release eggs into individual 0.5-liter containers. The females were then placed in the container with similarly staged eggs from the other female. The female and eggs were left undisturbed under cover for 3 h. In nearly all instances, the female retrieved these foreign eggs and was then housed in isolation in 1.5-liter tanks with constant flow and filtration. On day 13 of cross-fostering, affiliation experiments were conducted. The mature fry were removed from the buccal cavity of the female and placed in a white bucket (38 cm diameter, 48 cm high), filled to 15 cm with fresh Tanganyika-conditioned water. The female was then added to the test bucket. After 60 min, a digital image was taken and processed with ImageJ software to mark the location of each fry and of the mother in order to measure distances between them and calculate average distance between fry (social affiliation) and average distance between mother and fry (maternal affiliation). These measures were compared for fry of each stock that had developed in the buccal cavity of their own mother, versus those that had been cross-fostered in the buccal cavity of a mother from the other stock. All fry, regardless of stock or brooding condition, showed similar social affiliation, but WS fry showed greater maternal affiliation (Fig. 5). This result suggests that the behavior of fry may contribute to what has been termed 'good-mother phenotype'. These observed behavioral differences between the LS and the WS may involve

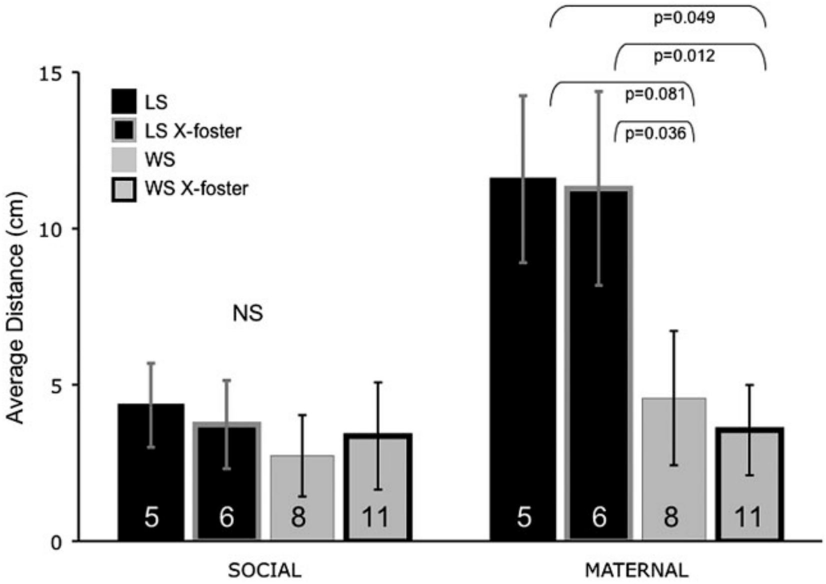

Figure 5 Stock differences for social behavior of fry. Average distance between individual fry quantifies social affiliation and average distance between mother and all fry in a brood quantifies maternal affiliation for LS (black bars) and WS (gray bars) fry brooded by their own mother or cross-fostered by a female of the other stock (bar outline colors indicate maternal stock), LS (black), and WS (gray). Measurements were made 1 h after fry removal from buccal cavity 1–4 days after initial release. Shorter distances correspond to greater affiliation. Error bars denote standard error, numbers denote sample size, *P*-values are indicated below braces.

differences in kin recognition and social bond formation.

Revisiting the social dominance of males in *A. burtoni* WS

Given the dramatically different behavior observed for the new WS with regard to behavior of the female and of the fry and also anecdotal observations of elevated aggression among WS males (i.e. great occurrence of injury in stock tanks), we questioned whether dominance behavior of males might also differ between the stocks. For each stock, we established observation groups (n = 3) of six males and six females in 30 gallon tanks with terra cotta pot shards to serve as territories. Using 5-min focal observations, the observer, blind to stock identity, quantified dominant and subordinate behaviors for each tagged males 2–3 times/week between 10:00 AM and 1:00 PM using the standard ethogram (Fernald 1977) adapted to the freely available event recorder, J-Watcher (Blumstein and Daniel 2007).

Given general fluctuations in social structure, the average percentage of dominant males across the three LS and three WS tanks over a 4-week period was not different with 37% dominant males in each WS tank and 32% dominant males in each LS tank. On average, a dominant WS male executed more chase behaviors than did a LS male (WS: 6.85 ± 1.22; LS: 3.34 ± 0.71; *t*-test: $P = 0.025$) (Fig. 6). However, there was no significant difference in the average occurrence of fleeing behavior between the LS and WS subordinate males (WS: 5.52 + 0.09; LS: 4.73 + 0.08; *t*-test: $P = 0.168$), suggesting that some of the observed aggressive chasing behavior in the WS tanks was directed at other dominant males (or females). When considering social interactions that occur predominantly between dominant males, the dominant LS males engaged in more border fights than did the dominant WS males (WS: 0.098 ± 0.040; LS:1.46 ± 0.29; *t*-test: $P = 0.00025$), but LS dominant males performed fewer lateral displays than did the dominant WS males (WS: 0.733 ± 0.289; LS: 0.077 ± 0.035; *t*-test: $P = 0.046$). This suggests that while overall levels of aggression are not different between the two stocks, the males of each stock use a different suite of behaviors in maintaining the dominance hierarchy, with a greater escalation among the LS males and greater use of ritualized threat in the WS males. Further individual observation and measurement of variation between individuals is warranted.

Behavioral differences within the dominant phenotype of males have been previously identified. Males are more likely to defend their territory against males of the opposite coloration (blue vs. yellow), indicating that coloration acts as a social signal (Korzan and Fernald 2005). Furthermore, in a

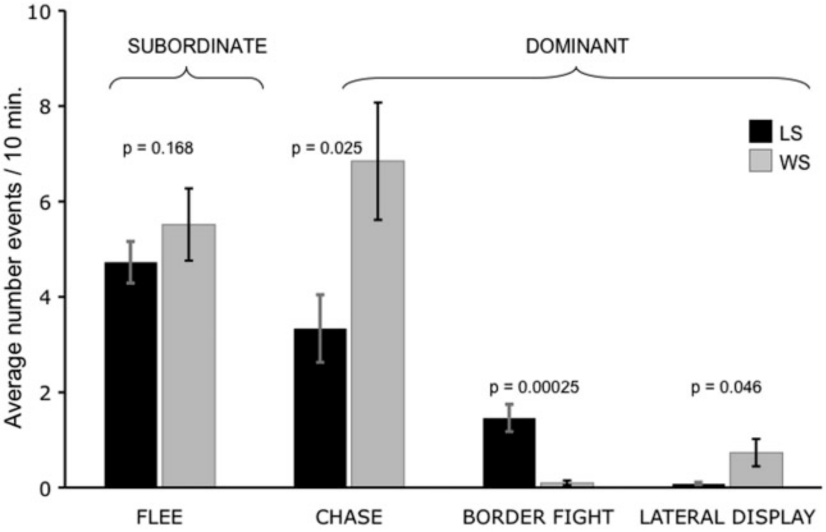

Figure 6 Stock differences for male social behavior. Average number of behavioral events per 10-min observation period. Flee is reported for subordinate males while aggressive behaviors of chase, border fight and lateral display, are reported for dominant males in LS (black) and WS (gray) populations (six males and six females per tank). The average number of events for 4 observation periods is reported only for males that displayed consistent dominance phenotype for 14 days. Error bars denote standard error.

contest between two dominant males, one yellow and one blue, the yellow male was more likely to obtain dominant status. In a more recent study, Korzan et al. (2007) also described an increased probability subordinate yellow, as opposed to blue, for males to ascend to dominant status, and an increased probability that a dominant yellow male would gain dominant status over a previously dominant blue male. Both studies were performed with the LS. Given that color is a plastic trait and not genetically fixed, the WS provides an opportunity to further investigate individual variation and behavioral differences among the color morphs of dominant WS males.

Conclusions

While it is possible that *A. burtoni* at the north end of the lake (i.e. LS) and at the south end of the lake (i.e. WS), have different behaviors in nature, it is more likely that 30 years of inadvertent artificial selection and inbreeding has led to the observed behavioral difference in the LS. The stock differences that have been observed thus far encompass behavior in males, females, and fry as well as physiological differences measured in females. The most striking difference between the stocks is the increased occurrence of maternal care and reduced filial cannibalism observed in the females of the recently collected WS. We suggest that the additional loss of body mass, which occurs in the LS females during brooding or during enforced starvation, may contribute to maternal cannibalism; however, controlled experiments that monitor body condition more closely are necessary in order to fully test this hypothesis. It is also possible that the increased maternal affiliation that is seen among the fry of the WS may contribute to the decreased cannibalism in that the females may be responding to the behavior of the fry.

While intentional selection for behavioral traits under laboratory, captive, or otherwise artificial environments can clearly lead to either increased (e.g. *Betta splendens*: Verbeek 2007) or decreased (foxes: Trut et al. 2004) levels of aggression, many other behavioral or physiological changes may also occur in captive populations. Among *Drosophila*, laboratory lines have been shown to diverge behaviorally, even beginning to show post-zygotic isolation (Boake et al. 2003). Similarly, behaviors such as predator avoidance (*Salmo turtta*: Alvarez and Nicieza 2003) and feeding strategy (*Danio rerio*: Robinson and Rowland 2005) can diverge in domesticated stocks through inadvertent selection or pleiotropic effects of intentional selection. Researchers are currently applying methods that were developed in model genetic systems to species that have not previously been examined genetically (e.g. Boake et al. 2002; Greenspan 2004). Often this effort requires the use of captive populations of less traditional model organisms (e.g. voles: Ophir et al. 2008), or the use of wild, or recently wild, populations of genetic model organisms (e.g *Drosphila*: Lavagnino et al. 2008; mice: Kimchi et al. 2007; *Xenopus*: Tobias et al. 1998). The stock differences described here for *A. burtoni*, after only 40 generations of captive breeding, even without intentional selection, not only highlight the necessity to consider the selective pressure of a laboratory environment, but also demonstrate the potential value of a comparative approach that contrasts behavior between strains.

In addition to these behavioral and physiological measures, these two stocks provide an opportunity to investigate the genetic and genomic basis for the observed behavioral differences. Therefore, while the cichlid system offers a wealth of fascinating behavioral and morphological diversity that was recently discussed at the symposium 'Genomics and Vertebrate Adaptive Radiation: A Celebration of the First Cichlid Genome' (Hulsey and Renn, 2009), the rapidly advancing genomic tools will not only contribute to the investigation of phenotypes in a comparative sense but will also contribute substantially to an in-depth understanding of a few key model species in this group. *A. burtoni* will continue to be an important model system for studying the mechanisms underlying socially mediated behavioral change.

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