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Forebrain AVT and courtship in a fish with male alternative reproductive tactics

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ABSTRACT: In this paper, we present the results of cellular and molecular studies on the neuroendocrine correlates of male sexual polymorphism in a population of the blenniid fish *Salaria pavo* (Risso). Bigger and older males defend nests, whereas smaller and younger males mimic female nuptial coloration and behavior to gain access to nests and sneak fertilizations. In this population, sex-role reversal in courtship also occurs (i.e., females are the courting sex). Immunocytochemistry and *in situ* hybridization were used to examine the production of arginine vasotocin (AVT) peptide and messenger RNA, respectively. The expression of AVT mRNA on a per-cell basis was correlated with mating behavior, rather than with sex morphotype, which suggests that the greater level of AVT mRNA expression in females and sneakers is correlated with the production of courtship behavior. On the other hand, the number and size of AVT peptide-producing cells in both male types is higher or larger, respectively, than in females, which suggests that it is correlated with sex morphotype, rather than with mating behavior. © 2002 Elsevier Science Inc.

KEY WORDS: Sex-role reversal, Sexual polymorphism, Sex differences, Neuropeptides.

INTRODUCTION

Arginine vasotocin (AVT) is a peptide hormone produced by the neurohypophysis that has been implicated in the regulation of the expression of courtship behaviour in a variety of nonmammalian vertebrates [9]. In species with male alternative reproductive tactics (ART), bourgeois males (i.e., males that compete among themselves for the attraction of mates) can be predicted to have higher AVT levels than can parasitic males (i.e., males that use noncourting mating behaviors to try to achieve sneaking fertilizations) [10], because the bourgeois males are usually the courting morph. In the three species with ART for which AVT data are available so far, bourgeois males have either larger soma of AVT-immunoreactive (ir) neurons or larger numbers of AVT mRNA producing cells in the preoptic area (POA) than do parasitic males (*Porichthys notatus*, *Thalassoma dupery*, and *T. bifasciatum*) [3,5, 7]. However, these differences can be attributed either to the differences in the expression of alternative morphotypes or to the differences in the expression of courtship behaviour between morphotypes. Therefore, neuroendocrine correlates of ART can only be established in species in which the expression of the bourgeois tactic is decoupled from the expression of courtship behaviour. This is the case of a population of the peacock blenny *Salaria pavo* living in a coastal lagoon in Southern Portugal, in which the scarcity of nest sites has lead to sex-role reversal in courtship behavior [1]. Males of this population do not leave the nests to court because of the high risk of nest takeover by floater males, and females initiate courtship, by displaying a distinct nuptial coloration and behaviour [1]. In addition, a proportion of smaller and younger males lack male secondary sex characters and mimic both the nuptial coloration and the courtship behaviour of the females, to gain access to nests and sneak fertilizations [6]. Thus, in this population, sneaker males are the courting male type.

In this paper, we present the results of cellular and molecular studies on *S. pavo* concerning the neuroendocrine correlates of this dramatic sexual polymorphism. We used immunocytochemistry and *in situ* hybridization to examine the production of AVT peptide and messenger RNA, respectively.

MATERIALS AND METHODS

Animals were caught in the intertidal areas of the Ria Formosa coastal lagoon (Ria Formosa Nature Park, Portugal) during low tide. All individuals were caught during the breeding season of 1996 (June to July). After capture, the animals were anaesthetized with MS-222 (Sigma Chemical Co., St. Louis, MO, USA) and perfused transcardially, with a marine teleost Ringer solution followed by a solution of 4% paraformaldehyde in 0.1-M phosphate buffer. The brains were extracted from the skull and stored until processing following the protocol described by Grober and Bass [8]. Brains were cryosectioned at 20 μm onto a series of three chrom-alum coated slides, which were stored with dessicant at -80°C.

For the AVT immunocytochemistry (ICC) procedure, the slides were treated using the methods outlined in Grober and Bass [8], except for the following details: (1) a Streptavidin-Biotin Kit (Gaithersburg, MD, USA) (KPL) was used and (2) a polyclonal antibody against AVT (kindly provided by Lieve Moons, Zoological Institute, Leuven, Belgium) was used at a concentration of 1:1000. Overnight pre-absorption of the antiserum with 1-μg/ml synthetic AVT (Sigma) eliminated all immunostaining.

For the *in situ* hybridization (ISH) procedure, we used the methods described by Godwin et al. [5]. This *in situ* hybridization method and the use of ^{33}P as a label generates very low background silver grain densities, and cells displaying specific labeling can be designated unambiguously. Following *in situ* hybridization and exposure of the emulsion, we quantified the size and number

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TABLE 1

DIFFERENCES (MEAN ± SEM) IN POA AVT CELLS AMONG MORPHOTYPES IN THE PEACOCK BLENNY

	Nest-holders	Sneakers	Females	Juveniles	ANOVA
AVT-ir cell size (μm^3)	43.9 ± 3.2 (4) ab	49.0 ± 4.7 (4) a	73.5 ± 12.6 (6) a	24.3 ± 10.9 (5) b	$F = 5.84$ $p < 0.01$
AVT-ir cell size/SL	5.28 ± 1.3 (4) a	8.3 ± 1.4 (4) ab	13.1 ± 5.3 (6) b	4.9 ± 6.9 (5) a	$F = 3.35$ $p = 0.048$
AVT-ir cell number	73.9 ± 45.1 (5) a	68.8 ± 48.8 (5) a	18.5 ± 17.6 (6) b	1.8 ± 2.7 (5) b	$F = 5.88$ $p < 0.01$
AVT-ir cell number/SL	8.3 ± 5.0 (5) a	11.7 ± 6.5 (5) a	3.4 ± 3.2 (6) b	0.5 ± 0.8 (5) b	$F = 6.73$ $p < 0.01$
Parvo-AVT-ir cell size (μm^3)	7.5 ± 7.5 (3) a	34.2 ± 11.0 (5) b	3.0 ± 7.2 (6) a	ND	$F = 5.25$ $p < 0.03$
Parvo-AVT-ir cell number	33.0 ± 20.7 (3) a	40.2 ± 14.4 (5) a	0.2 ± 0.4 (6) b	ND	$F = 4.02$ $p = 0.049$
ISH AVT-cell size	2214.7 ± 634.4 (6)	2051.4 ± 282.7 (5)	1554.1 ± 104.9 (4)	ND	NS
ISH AVT-cell number	18.7 ± 8.2 (6)	17.2 ± 10.3 (5)	22 ± 14.5 (4)	ND	NS
Density	33.8 ± 5.6 (6)	63.1 ± 3.9 (5) b	62.8 ± 1.8 (4) b	ND	F = 12.84 $p = 0.001$

Sample sizes are indicated between parentheses. Different letters stand for significantly different groups using a post-hoc test ($p < 0.05$). ND, not determined; NS, nonsignificant ($p > 0.10$), AVT, arginine vasotocin; ANOVA, Analysis of Variance; SL = Standard length; ISH, *in situ* hybridization.

of cells expressing AVT peptide and mRNA and the density of silver grains as a measure of mRNA expression.

Slides were coded so that the individual who quantified the different measures did not know the identity of the fish to which each slide corresponded. Sections were quantified using Power Macintosh 8500/120 (Apple, Cupertino, CA, USA), Scion image v.1.62a (Frederick, MD, USA), Microsoft Excel (Redmond, WA, USA), and Zeiss Axioskop light microscopy (Thornwood, NY, USA). Cell size was measured by capturing images and tracing the outside margin of the soma using National Institutes of Health (NIH) Image 1.55 (W. Rasband, NIH, Bethesda, MD, USA).

RESULTS

No significant differences were found between the adult male morphotypes neither in the mean cell size of POA AVT-ir neurons nor in its numbers (Table 1). Females had POA AVT-ir cells of similar size as males and larger than those of juveniles (Table 1). Both females and juveniles had significantly less POA AVT-ir cells than had males of both types (Table 1).

If juveniles are excluded (because of their extremely small size), a significant negative regression is found between cell size and standard length (SL) ($R^2 = 0.11$, $p = 0.009$) and a near-significant positive regression between AVT-ir cell number and body size (SL) ($R^2 = 0.23$, $p = 0.06$), which suggests that the comparison among morphotypes should be corrected for body size (SL). Even after correction for SL, no significant differences were found between the two male types, neither in cell size nor in cell number (Table 1). AVT-ir cell size corrected for SL was larger in females than in juveniles and nest-holders, and juveniles had fewer SL-corrected AVT-ir cells than did sneakers and nest-holders, and sneakers had greater numbers of neurons than did females (Table 1).

We have also compared the size and number of cells in the parvo-, magno-, and giganto-cellular components of the POA, following the nomenclature of Braford and Northcutt [2]. The only significant differences are that males and sneakers have large numbers of parvo-cells, whereas females lack these cells almost completely, and that sneakers have very large parvo-cellular neurons relative to nest-holders and females (Table 1). Because sneakers are younger than nest-holder males and breeding females, this suggests that the parvo-cellular area may undergo an explosive development early in life and then levels out over the following years.

The analysis of the *in situ* hybridization results was done for all cell groups combined, because we did not have enough data to look at each component of the POA independently. No significant differences were found in either the number or size of ISH-labelled areas between the three adult morphs (Table 1). Because of the spread of silver grains over areas that encompass multiple cells, it is not informative to compare the ICC cell size or numbers with the ISH areas or numbers from those same animals. The ISH density, that is, a measure of AVT mRNA expression per cell averaged across all cells, is highest in both females and sneakers relative to nest-holder males (Table 1).

DISCUSSION

The results presented herein show that the expression of AVT mRNA on a per-cell basis was correlated with mating behaviour, rather than with sexual morphotype. Female-like sneaker males and females produced about two times more AVT mRNA per cell relative to nesting males. Because sneaker males and females are the morphs that exhibit courtship behaviour in this population, we suggest that the greater level of AVT mRNA expression is correlated with the production of courtship behaviour.

The number and size of AVT-ir cells appears to be correlated with sexual morphotype, rather than with mating behavior, which is in accordance with published data for other species with ART [4]. Both male morphs had three times more cells than did females, but on average, these cells were only half as large as in females. Juvenile fish tended to have few small cells relative to any of the adult sexual morphs. Thus, ICC shows the conserved pattern of a sexual dimorphism, whereas the generation of alternative female

(sex-role reversal) and male behaviours (sneaking) is associated with an increase in AVT production per cell in *S. pavo*.

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REFERENCES

1. Almada, V. C.; Gonçalves, E. J.; Oliveira, R. F.; Santos, A. J. Courting females: Ecological constraints affect sex roles in a natural population of the blenniid fish *Salaria pavo*. Anim. Behav. 49:1125–1127; 1995.
2. Braford, M. R.; Northcutt, R. G. Organization of the diencephalon and pretectum of the ray-finned fishes. In: Davis, R. E.; Northcutt, R. G., eds. Fish neurobiology, vol. 2. Ann Arbor, MI: University of Michigan Press; 1983:117–163.
3. Foran, C. M.; Bass, A. H. Preoptic AVT immunoreactive neurons of a teleost fish with alternative reproductive tactics. Gen. Comp. Endocrinol. 111:271–282; 1998.
4. Foran, C. M.; Bass, A. H. Preoptic GnRH and AVT: Axes for sexual plasticity in teleost fish. Gen. Comp. Endocrinol. 116:141–152; 1999.
5. Godwin, J.; Sawby, R.; Warner, R. R.; Crews, D.; Grober, M. S. Hypothalamic arginine vasotocin mRNA abundance variation across sexes and with sex change in a coral reef fish. Brain Behav. Evol. 55:77–84; 2000.
6. Gonçalves, E. J.; Almada, V. C.; Oliveira, R. F.; Santos, A. J. Female mimicry as a mating tactic in males of the blenniid fish *Salaria pavo*. J. Mar. Biol. Ass. U.K. 76:529–538; 1996.
7. Grober, M. S. Socially controlled sex change: Integrating ultimate and proximate levels of analysis. Acta Ethol. 1:3–17; 1998.
8. Grober, M. S.; Bass, A. H. Neuronal correlates of sex/role change in labrid fishes: LHRH-like immunoreactivity. Brain Behav. Evol. 38: 302–312; 1991.
9. Moore, F. L. Evolutionary precedents for behavioral actions of oxytocin and vasopressin. Ann. N.Y. Acad. Sci. 652:156–165; 1992.
10. Taborsky, M. Bourgeois and parasitic tactics: Do we need collective, functional terms for alternative reproductive behaviours? Behav. Ecol. Sociobiol. 41:361–362; 1997.