Evolutionary pattern in the OXT-OXTR system in primates: Coevolution and positive selection footprints

Pedro Vargas-Pinilla1,2, Vanessa Rodrigues Paixão-Côrtes1,2, Pamela Paré2, Luciana Tovo-Rodrigues2, Carlos Meton de Alencar Gadilha Vieira3, Agatha Xavier3, David Comas3, Alcides Pissinatti3, Mariaíla Sinigaglia3, Maurício Menegatti Rigo3, Gustavo Fioravanti Vieira3, Aldo B. Lucion3, Francisco Mauro Salzano1,2, and Maria Cátira Bortolini1,2

*Departamento de Genética, Instituto de Bionciências, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, RS, Brazil; 1Instituto de Biologia Evolutiva, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, 08003 Barcelona, Spain; 2Centro de Primatologia do Rio de Janeiro, 20940-200 Rio de Janeiro, RJ, Brazil; and 3Departamento de Fisiologia, Instituto de Ciencias Básicas da Saúde, Universidade Federal do Rio Grande do Sul, 90050-170 Porto Alegre, RS, Brazil

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Oxytocin is a nonapeptide involved in a wide range of physiologic and behavioral functions. Until recently, it was believed that an unmodified oxytocin sequence was present in all placental mammals. This study analyzed oxytocin (OXT) in 29 primate species and the oxytocin receptor (OXTR) in 21 of these species. We report here three novel OXT forms in the New World monkeys, as well as a more extensive distribution of a previously described variant (Leu8Pro). In structural terms, these OXTs share the same three low-energy conformational solutions in solution during molecular dynamic simulations, with subtle differences in their side chains. A consistent signal of positive selection was detected in the Cebidae family, and OXT position 8 showed a statistically significant (P = 0.013) correlation with litter size. Several OXT changes were identified, some of them promoting gain or loss of putative phosphorylation sites, with possible consequences for receptor internalization and desensitization. OXTR amino acid sites are under positive selection, and intramolecular and intermolecular coevolutionary processes with OXT were also detected. We suggest that some New World monkey OXT-OXTR forms can be correlated to male parental care through the increase of cross-reactivity with its correlated vasopressin system.

OXT | OXTR | primates | coevolution | behavior

Oxytocin has crucial functions related to physiological processes and social behaviors in primates and other placental mammals. A nonapeptide (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly) (1), oxytocin (OXT-8Leu) is both a neurotransmitter released by neuronal cells in synapses and a hormone, activating receptors distant from the site of its synthesis through the circulatory system (2). In mammals, OXT acts as a hormone in uterine contraction during parturition and in milk ejection while lactating. It is also a key central nervous system neurotransmitter, regulating/modulating complex social and reproductive behaviors (i.e., pair bonding and parental care) (3–7).

Until recently, it was believed that the OXT amino acid chain was the same in all placental mammals. However, Lee and colleagues (8) reported a T > C change in four New World monkeys (NWms), Saimiri sciureus, Cebus apella, Callithrix jacchus, and Aotus nancimae, substituting leucine to proline at position 8 (OXT-8Pro). This form was also found in Tupaiia belangeri, a tree shrew species of Southeast Asia (8). OXT differs from its paralog vasopressin (AVP; Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly) at positions 3 and 8. Variation at position 8 also identifies nonplacental OXT/AVP-like nonapeptides, such as mesotocin, present in some marsupials (7, 9). These findings dispel the notion of a universal OXT amino acid sequence for placental mammals.

OXT activity depends on adequate interaction with its unique receptor, OXTR, although it can also bind to the vasopressin receptors (AVPR1a, AVPR1b, and AVPR2) with lower affinity (11–13). Similar to other receptors that use G proteins as transducer signals across the cell membranes, OXTR is composed of seven transmembrane (TM1–TM7), four extracellular (N-terminal tail-ECL3), and four intracellular (ICL1-C-terminal tail) domains. ECL and ICL are important for the interaction with OXT and G proteins, respectively, whereas TMs are connected with both functions (7, 11).

In contrast to what is observed for placental mammal OXT, OXTR presents hundreds of variants in regulatory and coding regions, including at the intraspecific level. In humans, OXTR single-nucleotide polymorphisms have been associated with several social behavioral phenotypes (14).

The presence of OXT-OXTR-related systems throughout the animal kingdom indicates that their typical roles in placental mammals are likely exaptations of ancient functions, such as regulation of fluid balance and egg-laying (15, 16). Studies have attempted to investigate both the interaction of OXT-OXTR-like systems and their coevolution (11, 17). However, our knowledge about this nonapeptide-receptor system, including the extent of its variability in the primate order, remains limited.

Significance

It was previously believed that placental mammals present no variability in oxytocin (OXT). The present study reports novel data on the diversity of OXT and its receptor (OXTR) in primate species, including New World monkeys. Contrary to prior expectations, we found three novel OXT forms and several OXTR nonsynonymous changes not previously described. In the Cebidae family, signals of positive selection were found for an OXT variant at position 8, which is associated with larger litter sizes. We detected positive selection for OXT forms and report a coevolutionary process between changes in OXT and OXTR.


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1P.V.-P. and V.R.P.-C. contributed equally to this work.
2To whom correspondence may be addressed. Email: francisco.salzano@ufrgs.br or maria.bortolini@ufrgs.br.

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NWm emerged ~30 million years ago. They are classified into 16 genera and ~75 species and present a wide range of reproductive and social behaviors (18, 19), but little is known about their genetic variability and concurrent phenotypic variation (20).

The present study reports results about OXT and OXTR diversity in 29 primate species, including 20 NWm species. These analyses include original OXT and OXTR sequences for 16 and 12 NWm species, respectively. We discuss details about the co-evolution of these systems, as well as possible connections among reported genetic variability, positive selection, and some key species-specific biologic traits.

Results

OXT Variants in Primates. Fig. 1 shows that OXT-8Pro is present in all species from the Cebidae family; furthermore, A > G (isoleucine > valine) at position 3 (OXT-3Val-8Pro) in Saguinus. The Pithecidae family presented two novel OXT forms, both with changes at codon 8: C > G (leucine > alanine) in Cacajao melanophaeus and Chiroptes utahickae (OXT-8Ala), and C > A (leucine > threoneine) in one specimen of Chiroptes albinasus (OXT-8Thr).

All amino acid changes were found to be homozygous. In species tested with multiple individuals, no intraspecific variation was found, suggesting these OXT forms are probably species- or group-specific.

Grantham scores (GSs) were calculated to investigate the putative functional effects of detected changes in OXT. Cebidae OXT8-Pro and Pithecidae OXT8-Ala, as well as C. albinasus OXT-8Thr, are moderately conserved (GS = 98, 96, and 92, respectively), whereas a Val at position 3 in Saguinus is a conservative change (GS = 29). No statistically significant differences in relation to biochemical properties were found using PRIME and SIFT tools.

The available 3D structure for OXT (PDB ID code 1NPO) (21) was used to perform a structural evaluation of the implications of these changes. OXT-8Leu was in silico with water and ions at specific physiologic concentrations in a virtual cube. On the basis of this molecular dynamics simulation, a free-energy

![Oxytocin nucleotide and amino acid sequences observed in primates, with the OXTR positions coevolving with OXT. The maximum likelihood phylogenetic tree was based on an ~8-Mb genomic sequence reported by Perelman and colleagues (19). Nonsynonymous nucleotide and amino acid changes are shown in red. Taxonomic families and subfamilies are indicated, as well as species with common male care (in bold) and those with twin pregnancies (asterisks). The trace (−) indicates that no information is available.](image)

Fig. 1. Oxytocin nucleotide and amino acid sequences observed in primates, with the OXTR positions coevolving with OXT. The maximum likelihood phylogenetic tree was based on an ~8-Mb genomic sequence reported by Perelman and colleagues (19). Nonsynonymous nucleotide and amino acid changes are shown in red. Taxonomic families and subfamilies are indicated, as well as species with common male care (in bold) and those with twin pregnancies (asterisks). The trace (−) indicates that no information is available.
Surface analysis revealed three main low-energy conformations: type 1, type 2, and type 3 (SI Appendix, Figs. S1A and B and S2 and Table S1). Type 1 has a more open conformation than the others, whereas type 2 and type 3 are more compact. Type 2 also shows hydrogen bonds between the Tyr2/Cys6 and Tyr2/Asn5 residues, which lead to the typical ring structure that resembles oxytocin complexed with neurophysin, its molecular carrier (21, 22). All OXT forms showed the presence of the same three conformational types, with some small differences in the side chains (SI Appendix, Fig. S1A).

The same experiment was conducted with AVP. SI Appendix, Fig. S1B shows that these three conformational types are also observed there, with small changes in the side chains. This suggests preservation of the 3D conformations that allow nonapeptide transport (through connection with neurophysin) and adequate coupling with receptors.

It is difficult to imagine these OXT changes have no evolutionary implications. Thus, we tested whether they might have been under selection through two different approaches. The NsSites model failed to detect codons under positive selection. However, when considered site by site, a nonsynonymous/synonymous ratio (ω) of ~1 was found for OXT at position 8. This site was also recovered by the Branch-site model, indicating relaxation (SI Appendix, Fig. S3). This last model detected evidence for positive selection in the Cebidae family. Clade Model D had a better performance compared with the neutral model, using a likelihood ratio test (P = 0.0368 ω = 125.99; SI Appendix, Table S2). This result indicates that the primate ω values can vary between branches, suggesting a distinctive evolutionary pattern for the Cebidae family.

In addition, when the species phylogenetic tree (Fig. 1) is visually compared with the OXT maximum likelihood tree, different topologies can be clearly observed (SI Appendix, Fig. S7). When an incongruence of this nature is detected, a simple neutral model of mutation and drift is insufficient to explain the pattern found.

We assessed significance associations between molecular OXT forms and specific NWM social behavior and ecological traits, using Mann-Whitney or Fisher exact tests (23). Proline at position 8 was significantly associated with larger litters (P = 0.013), but not with other ecological/social primate traits (SI Appendix, Tables S3 and S4).

**OXTR.** Our analyses reveal 73 nonsynonymous OXTR changes, 17 of which have not been previously described, to our knowledge (SI Appendix, Table S5), whereas others are located in known human polymorphic sites. Of the 73 sites, 18 (five novel) are located in the N-terminal tail, one of the most relevant domains for the interaction with OXT. Thirty-eight changes (six novel) are present in the intracellular domain 3 and C-terminal tail, and 10 (six novel) are found along TM1 and TM3–TM5 (SI Appendix, Fig. S4). The highest number of nonsynonymous changes was found in Cebus xanthosternos, Saginus niger, and C. utahickae (27 each), and S. sciureus and C. utahickae have the higher number of singletons (four each).

According to the ω, 4% of the OXTR changes are considered radical changes: Gly22Trp (located at the N-terminal tail; GS = 184), Trp161Gly (TM4; GS = 184), and Cys383Gly (C-terminal tail; GS = 159). Twenty percent of the changes lead to moderately radical changes and are present in the N-terminal tail, TM1, ICL2, and C-terminal domains. The amino acid change at position Trp161Gly (C. xanthosternos) was also predicted as damaging, using SIFT (SI Appendix, Table S5).

Although homology modeling and docking experiments are important for understanding the OXTR-OXT system interaction, the G protein coupled receptor (GPCR) signaling within intracellular domains is also fundamental to elucidating receptor response. On this basis, we analyzed the level of OXTR protein disorder, secondary structure, and solvent accessibility, as well as the putative phosphorylation sites in the intracellular domains. Our analyses indicated that OXTR has long disordered regions on N, C-terminal tails and a portion of ICL3. The analyses showed that the changes do not introduce any important difference in flexibility or accessibility. Taking into account the available information for two GPCRs (protein kinase C and the G protein coupled receptor kinase), 13 putative phosphorylation sites were identified (SI Appendix, Table S6).

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In addition, the Selecton software detected three positively selected OXTR sites, 6Ala (N-terminal) and 255Val and 251Gly (ICL3), two of which NsSites also detected. All are located in important regions that interact with the nonapeptides or G proteins (SI Appendix, Fig. S4).

These results reveal that the degree of conservation and evolutionary rates were unequally distributed across the OXTR domains. The N-terminal and ICL3 regions were most variable, whereas TMs were most conserved. This may be because TMs have the double function of interaction with nonapeptides and G proteins (7, 11).

**OXT-OXTR Coevolution.** Intramolecular coevolution analyses (SI Appendix, Table S8) showed that C. geoffroyi, C. jacchus, Cebuella pygmea, C. xanthosternos, and C. albinasus are coevolving at OXT positions 3 (N-terminal) and 149 (ICL2). When a serine is found at position 23, a serine is also found at position 149 (posterior probability, 81%), creating a new putative phosphorylation site recognized by protein kinase C (SI Appendix, Table S6).

Other important results emerged regarding OXTR binding sites (Arg-34, Phe-103, Tyr-209, and Phe-284) (25–28). These sites are conserved in the species sampled, with the exception of S. sciureus, which showed the Phe103Tyr change (7). OXTR-103 is a binding site for OXT at position 8 (SI Appendix, Fig. S4) and a homolog of the binding site for AVPR1a at position 115. Remarkably, when the AVPR1a-115 site is artificially mutated in rat cells (Tyr115Phe), the affinity of AVPR1a for OXT increases (29). Keeping in mind that Tyr is the most common amino acid present in AVPR1a, the presence of a Tyr at OXT position 103 could increase its affinity for AVP at least in S. sciureus. These findings suggest that coevolutionary processes might involve not simply OXT and OXTR, or AVP and its three receptors, but rather, a cross-reaction between these systems.

The Spidermonkey software showed two coevolutionary processes in intermolecular analyses: OXT at position 3 coevolves with OXTR (posterior probability, 86%, C-terminal, posterior probability of 56%), whereas OXT at position 8 coevolves with OXTR at position 345 (C-terminal, posterior probability of 80%; SI Appendix, Fig. S4 and Table S8). More specifically, the three species of...
Saguinus have a Val at position 3 concomitantly with a Leu at OXTR 368. As mentioned, the latter change removes an important phosphorylation site from one known OXTR serine cluster, which could modify the desensitization of G protein-coupled receptors (11, 24).

In addition, a correlation analysis revealed that species that have the highest OXT genetic distances tend to show a similar pattern with OXTR (p = 0.52; P < 0.001; SI Appendix, Table S9), independent of their position on the phylogenetic tree. This result suggests similar evolutionary tendencies, which can be a result of natural selection.

Discussion

The nonapeptide oxytocin and its paralog vasopressin perform crucial functions in physiologic processes and social and reproductive behaviors in placental mammals. During the roughly ~70 million years of evolution that separate the human and mouse lineages, no amino acid replacement occurred in OXT. In contrast, NWms present five forms of OXT, with changes found exclusively at positions 3 and 8. These positions are the most important for highly selective binding to different receptor subtypes (29). Interestingly, AVP is conserved in the NWm branch (SI Appendix, Fig. S6), at least according to 15 species studied recently (30).

Prominent examples of positive selection in the NWm lineage are rare (20). Here we obtained consistent evidence for positive selection in OXT for the Cebidae branch (OXTR-8Pro and OXT-3Val-8Pro). For OXTR, we identified sites that seem to be relevant for the function of the OXT-OXTR system beyond those previously identified (25–28). For three of them (sites 6, 251, and 255), signals of positive selection were detected. Intermolecular coevolutionary analysis also revealed that Saguinus presents a Val at position 3 of OXT that seems to have coevolved with a Leu at 368 of OXTR. It is noteworthy that Smith and Ginsburg (31) demonstrated through in vivo and in vitro experiments that a Val at position 3 of OXT increases uterus contraction, indicating its functionality at least for this trait.

Because natural selection acts on phenotypes, we verified whether the amino acid changes described here would have phenotypic implications. Although the GS predictions for the new NWms-OXTs showed moderate/conservative values and no significant alteration in some physicochemical properties, all changes deserve to be investigated. For instance, comparing isotocin (present in fishes) (2) with mesotocin (found in birds, lungfish, reptiles, amphibians, and some marsupials; 2) identified two amino acid changes, Ser > Gln (GS = 68) and Leu > Ile (GS = 5), at positions 4 and 8, respectively. In addition, the difference between mesotocin and OXT consists of only one alteration at position 8: Ile > Leu (GS = 5). These changes have lower GS values than those we report here in the NWm group.

Furthermore, our analysis reveals that the five OXT forms present three preferential conformations in solution, suggesting the preservation of basal characteristics of these molecules and, consequently, of their primordial functions in placental mammals.

As for OXT-OXTR affinity, an instigating example of coevolutionary analysis can be cited: S. sciureus presents both OXT-8Pro and OXT-3Val-8Pro forms. The presence of the Tyr residue at position 103 could increase affinity with AVP at position 8, similar to what occurs with OXT and AVPR1a when changes are introduced in homolog sites (29). Considering receptor activation and signaling, a leucine at OXTR-368 in Saguinus (coevolving with a Val at OXT position 3) destroys an important phosphorylation site. This could lead to instability of the OXTR-β-arrestin interaction and consequent changes in OXTR internalization and desensitization. Also, a serine at OXTR position 149 (coevolving with a Ser at position 23 in several species) creates a new putative phosphorylation site recognized by protein kinase C.

The processes described here can be connected to ecological and social behavior traits, particularly in the Callitrichinae subfamily, where we found an association between larger litter size and a proline at position 8.

Both OXT and AVP are related to pair bonding and parental care, but apparently the OXT system plays a more important role in females and the AVP system primarily influences males. This suggests an interesting sexually dimorphic pattern (32), which has been described in C. jacchus (33). Another study showed that AVPR1a increases in dendritic spines of the prefrontal cortex during C. jacchus fatherhood (34). Furthermore, experimentation with prairie voles showed the importance of AVP and AVPR1a in complex male behaviors, including monogamy and paternal care (2, 35). Thus, our finding of the OXT-8Pro and OXT-3Val-8Pro in the Cebidae could increase OXT affinities to AVP receptors (especially AVPR1a). Meanwhile, changes in OXTR (i.e., OXTR-103Tyr) could increase its affinity to AVP.

In Callitrichinae, the subfamily of the Cebidae to which Saguinus and Callithrix belong, it is well documented that males (fathers, siblings, and unrelated) provide important support for infant survival (36). Studies also demonstrate that male exposure to newborns increases responsiveness for care in inexperienced alloparental Callitrichinae (37). There are well-known reasons that could justify this pattern of multiple helpers: first, the high energetic costs of reproduction (birth weight of twin infants is ~30% of Callitrichinae females’ body weight), and second, females have a postpartum estrus and may become pregnant while still nursing. The result is that infant survival rates of experienced mothers with inexperienced fathers and no siblings are lower than those observed when both parents are experienced and siblings are present (36). In addition, even in some Cebidae species in which twin births are rare, paternal care is present, which could be connected to a strategy that promotes the evolution of genetic monogamy, such as observed in Aotus azarae (38) and Callimico goeldii (20).

Paternal care seems to have evolved independently at least four times in the radiation of the primate order, one of which is in the Cebidae branch (39). Thus, OXT-8Pro and OXT-3Val-8Pro forms could influence behaviors through increase of affinity, at least with AVPR1a. These behaviors include parental male care in Cebidae species with twin pregnancies or in other reproductive and social circumstances in which male care (both parental and alloparental) is fundamental for adaptive success. Several studies reveal intense male and female parental, as well as alloparental, care in the Callitrichinae subfamily (20). Thus, Cebidae OXT-8Pro and OXT-3Val-8Pro forms can be also connected with female behaviors. Recently, Cavanaugh and colleagues (40) showed that treatment with OXT-8Pro in C. jacchus facilitated fidelity in females, but not in males, and they unfortunately did not test other behaviors, including parental care.

Curiously, the shrew T. belangeri presents both OXT-8Pro and OXTR-103Tyr, as observed in S. sciureus. The simultaneous presence of these two forms in different taxa suggests parallel evolution, providing a potential example of positive selection and adaptive evolution (41). T. belangeri males do not take care of their offspring. A similar behavior is observed in Saimiri, one of only two genera of the Cebidae family without paternal care (39), indicating a possible reversion from the ancestral state (Fig. 1). Another parallelism involves the OXTR Ala218Thr (652C > T) change found in S. sciureus. It is also reported as a human polymorphism (rs4686302), with implications in ligand-receptor affinity and preterm births (42). The implication of this change to S. sciureus is unknown.

Finally, although some species studied here share similar reproductive and parental care behaviors, they have also intra- and interspecies differences (43, 44). Thus, the general genotype-phenotype connections suggested in the present study should be considered with caution and can only be confirmed with

Vargas-Pinilla et al.
additional population and functional studies, as well as investigations with related genes.

**Conclusion**

We reported novel OXT forms in NWm and a previously unrecognized lack of sequence conservation in placental mammals. Signals of positive selection and of coevolution at inter- and intramolecular levels were detected. Some changes in the OXT-OXTR system seem to be connected to specific ecological/social behavior traits, such as intense parental care.

**Materials and Methods**

**DNA Samples, Sequencing, and Sequence Alignment.** Blood samples from 41 individuals belonging to 16 NWm species (SI Appendix, Table S10) were provided by Rio de Janeiro’s Primatology Center (mapadecultura.ri.gov.br/guapiirimirocentro-de-primatologia-do-rio-de-janeiro).

Genomic DNA was extracted and PCR products were purified and sequenced (27 and 1,167 nucleotides coding for the OXT nonapeptide and OXTR systems, respectively), using Applied Biosystem Genetic Analyzer sequencers (GenBank accession nos. KM186262 to KM186289).

DNA sequences from genomic databases were also included in the analyses (SI Appendix, Table S10), for a total of 29 primate species for OXT and 21 for OXT and OXTR.

Multiple alignments were performed in the Mega software (5.1 version), using amino acid sequences with the MUSCLE algorithm (45). The alignments were also visually checked.

**Evolutionary Analyses.** We used the phylogeny-based maximum likelihood analysis, as implemented in the CODEML program of the PAML 4.7 package, to test for positive selection and/or relaxation of functional constraints (46).

Two approaches using the nonsynonymous/synonymous ratio (dN/dS = ω, where ω < 1 indicates negative or constraint selection, ω ≥ 1 indicates neutral or relaxing selection, and ω > 1 indicates positive selection) were applied: the dN/dS codon substitution model, which allows ω values to vary among sites, and the Branch-site Models, which enable ω variation in different branches of the phylogeny. Unrooted trees, necessary for the construction of the input files, were created on the basis of the primate phylogenetic tree provided by Perelman and colleagues (19). Coevolution was tested at the intramolecular (within single molecules or genes) or intermolecular (between different molecules or genes) levels (47). First, we considered recognized specific binding sites, OXT amino acid chain positions 3 and 8, and OXTR amino acid chain positions 34, 103, 209, and 284 (25–28).

Second, we examined coevolutionary processes through the Bayesian Spidermonkey tool (48), available at the Datamonkey server (www.datamonkey.org/). This test furnishes the posterior probability of a change in a site as dependent on a change or changes in another site or sites. Intra- and intermolecular (inside the same gene) and intermolecular (between different genes) analyses were performed. Third, we calculated pairwise Nei-Gojobori (49) OXT/OXTR distances for the 21 primate species with MEGA 5.2. Correlation between genetic distance matrices was calculated using the Mantel test (GenALEX 6.5) (50).

**Molecular Characteristics and Predictive Functionality.** Changes found in NWm species were compared with the amino acid residue present in Otomlem garnetti and classified according to Grantha scores (51) as conservative (0–50), moderately conservative (51–100), moderately radical (101–150), or radical (>151) (52). Changes in physicochemical properties were also evaluated using PRIME (53) and SIFT (54).

The structures of OXT and AVP variants were modeled starting from the OXT-Bluelu crystal 3D structure (PDB ID code 1NPO). A molecular dynamics simulation was applied to the six structures (five OXT and one AVP) along 400 ns to evaluate the effect of amino acid changes in solution (two independent simulations for each system, using the GROMACS package v. 4.5.1) (55). To retrieve low-energy conformers, which can reflect stable conformations in solution, a Free Energy Surface was calculated for the OXT and AVP molecules. In addition, a clustering approach was applied to determine the most frequent conformational states of the simulation of each peptide hormone.

Although 3D crystal structures of GPCRs are known, they present less than 29% similarity with OXTR. This level of similarity can be used to model G protein-coupled A class receptors (13, 27). However, we opted for a more conservative approach to evaluate the OXTR changes. We took into account functional information for OXTR intracellular domains (loops and C-terminal portion) to search for phosphorylation posttranslational modifications, as they are very important for GPCR regulation, as well as dimerization (24, 56, 57). Three available software packages were used to obtain a consensus result: PSPP 1.06 Prediction of PK-specific Phosphorylation site (58), NetPhos 1.0 (59), and GPS 2.1 Group-based Prediction System (58). For predictions, only consensus sites for kinases experimentally known to interact with OXTR, namely, Protein Kinase C (60) and G protein-coupled receptor kinase (61), were considered. In addition, we analyzed the pattern of protein disorder, secondary structure, and solvent accessibility in the OXTR intracellular domains to assess the effect of changes on loop flexibility and to offer further support for phosphorylation analysis, using PONDR-FIT (intrinsic protein disorder) (62), PSIPRED and NetSurfP v.1.1 (secondary structure) (63, 64), and NetSurfP v.1 (solvent accessibility) (64).

**OXT/OXTR Forms and Ecological/Social Behavioral Traits Analysis.** We assessed significance associations between molecular OXT and OXTR changes and seven NWm social behavior and ecological traits (SI Appendix, Table S3), using Mann-Whitney or Fisher exact tests (23). Differences were considered significant with a P value < 0.05 after Bonferroni correction.

A complete description of all procedures listed here is found in the SI Appendix.

**Ethical Approval.** This project was registered in the official Brazilian system, which permits the collection of biological material for research in conservation units [Sistema de Autorização e Informação em Biodiversidade (SISBIO) number 27951–2].

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