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The Role of Prolactin in the Regulation of Brood Care in the Cooperatively Breeding Fish *Neolamprologus pulcher*

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ABSTRACT The hormone prolactin (PRL) is important for the regulation of parental care in many species of mammals, birds and fish, and for alloparental care (care directed at nondescendant young) in some mammals and birds. Its significance in alloparental brood care of cooperatively breeding fish has not yet been assessed. Here, we test the role of PRL in brood care behavior of the cooperatively breeding cichlid *Neolamprologus pulcher*. The expression of PRL mRNA was determined in the pituitary glands of breeders of both sexes, helpers that showed brood care behavior and nonbreeding fish as controls. In addition, PRL levels were experimentally manipulated in male breeders and helpers by intraperitoneal injections of ovine PRL, and the behavior of these test fish was recorded toward standardized clutches. Adult females had higher levels of PRL mRNA than adult males, which was true both for breeders and nonbreeders. Contrary to expectation, there was no positive correlation between PRL and brood care behavior in any category of test fish, and the experimental application of PRL did not change brood care propensity. Interestingly, brood-caring adult females had significantly lower levels of PRL mRNA than adult female nonbreeders, whereas there was no difference between helpers and similar-sized nonbreeding group members. PRL mRNA levels increased with body mass in juveniles, but decreased with body mass in adults. In conclusion, we found no evidence that elevated levels of PRL are directly involved in the regulation of brood care behavior in this species. *J. Exp. Zool. 309A:515–524, 2008.*

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Alloparental brood care denotes the care directed at nondescendant young (Wisenden, '99). Among vertebrates, it has been described for birds (Brown, '87; König and Dickinson, 2004), mammals (Solomon and French, '97; Clutton-Brock et al., 2002) and fish (Taborsky, '94, 2001; Wisenden, '99). The endocrine regulation of alloparental care is still poorly understood (Schoech et al., 2004), but a role for the versatile pituitary hormone prolactin (PRL) with over 200 reported biological functions (Freeman et al., 2000) is likely, particularly when the importance of PRL for brood care in mammals and birds is considered (for reviews, see Schradin and Anzenberger, '99; Ziegler, 2000).

In fish, PRL was primarily studied in the context of osmoregulation, but it also influences metabolism and behavior (Clarke and Bern, '80). In particular, PRL was found to be of major importance for the regulation of brood care (Blüm and Fiedler, '65; Slijkhuis et al., '84; Tacon et al., 2000; Páll et al., 2004). In species that do not show natural brood care behavior, the application of PRL alone does not elicit such behavior (Blüm, '74), but in species showing brood care it could be elicited or intensified by PRL. In some species this was only the case when other hormones such as gonadotropins, testosterone or progesterone were administered simultaneously (*Hemihaplochromis multicolour* and *Tilapia mossambica*, Bartmann,

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'68; *Macropodus opercularis*, Machemer, '71; *Lepomis gibbosus*, Kramer, '73). In other species, brood care experience and external stimuli such as the presence of a clutch are essential components to release brood care propensity after PRL administration (*Hemichromis bimaculatus*, Noble et al., '38; *Trichogaster trichopterus*, Kramer, '72; *L. gibbosus*, Kramer, '73; *Gasterosteus aculeatus*, Bruijn and Sevenster, '83). It is unclear which intrinsic or ecological characteristics of species might account for these interspecific differences.

Here, we studied the expression and the influence of PRL on parental and alloparental brood care behavior in the cooperatively breeding cichlid *Neolamprologus pulcher*.[1] This endemic substrate breeder of Lake Tanganyika, East Africa, is the most highly advanced social fish species known to date (Taborsky and Limberger, '81; Taborsky, '84; for a review, see Taborsky, '94, 2001; Balshine et al., 2001; Bergmüller et al., 2005; Brouwer et al., 2005; Dierkes et al., 2005; Stiver et al., 2005). *N. pulcher* breeds throughout the year without obvious seasonal variation. Sexually mature and immature helpers of both sexes participate in direct brood care (fanning, cleaning of eggs), defense, territory maintenance and cleaning of the breeding substrate, and it seems likely that variable regulatory mechanisms exist. Among members of a pair, the readiness of females to care for eggs depends more strongly on the stage in the reproductive cycle than it does in males (von Siemens, '90). This suggests a stronger role for reproductive hormones in the control of brood care behavior in the female sex. For both, breeders' and helpers' exogenous stimuli of the clutch are important for the maintenance of brood care behavior and for its ''fine tuning,'' and for helpers the dominance status is most influential (von Siemens, '90). A previous study in this species showed no difference in androgen levels of male breeders and male helpers in the presence or absence of a clutch, suggesting no strong influence of sex steroids on brood care behavior of males (Bender et al., 2006).

To our knowledge there is no information about the role of PRL in brood care behavior of alloparental fish. With this study we aim to fill this gap to contribute to the understanding of hormonal control mechanisms and the evolution of alloparental brood care behavior in vertebrates. We hypothesize that if PRL is important, fish with a clutch should have higher PRL levels and therefore increased PRL mRNA expression compared with fish with no clutch. This difference should be stronger in females than in males, as female breeders show the highest levels of direct brood care (Taborsky, '84). We also expected higher PRL expression levels in helpers showing brood care behavior than in same-sized juveniles not living in breeding groups.

MATERIALS AND METHODS

Experimental design

In order to establish whether pituitary PRL levels are correlated with brood care behavior in *N. pulcher*, PRL mRNA was measured in the pituitary glands of eight breeders of each sex and in nine immature helpers, which showed brood care behavior (family fish), and in similar-sized nonbreeding fish living in groups that served as controls (aggregation fish, eight males, eight females and ten juveniles).

In order to establish whether PRL influences brood care behavior, circulating PRL levels in test fish (male breeders and helpers at the onset of maturity) were manipulated and their behavior toward foreign test clutches was observed. The presence of a foreign test clutch has been shown to induce brood care behavior in subordinate fish and in male breeders of *N. pulcher* outside of a breeding cycle (von Siemens, '90).

Maintenance of animals

Our experimental fish were descendants of wild-caught fish from the southern end of Lake Tanganyika (caught close to Mpulungu, Zambia). They were kept at the Ethologische Station Hasli, Institute of Zoology, University of Bern. Experimental tanks contained 60 L (for PRL mRNA measurements) or 100 L (for injection experiments with subsequent observation) of freshwater, respectively, and were structured with a layer of sand and two halves of a bottomless flowerpot as shelters for hiding and breeding. Family groups consisted of a breeder pair, several helpers of different sizes and fry. Aggregations of nonreproducing fish, which also occur in the wild (Taborsky and Limberger, '81; Taborsky, '84), consisted of adults of both sexes and juvenile fish. They were kept in groups in tanks of 200 or 400 L containing freshwater and a sand layer but no shelters so that territoriality and breeding did not occur. Fish

[1] *N. brichardi* and *N. pulcher* have been described as two separate species, but there is morphological and genetic evidence that they should be referred to as one species only; see Grantner and Taborsky ('98) and Duftner et al. (2007).

were always observed and manipulated at the same time of the day (13.00–15.00 hr) to avoid a potential effect of diurnal changes in behavior and hormone levels. They were fed ad libitum in the evening with Tetramin (Melle, Germany) dry food four times a week and with frozen mosquito larvae twice a week. The social status, sex, size and mass of each fish were determined before each manipulation. All observations and manipulations of fish adhered to the official rules and regulations of Switzerland about handling experimental animals and were approved by the veterinary council of the Kanton Bern.

Measurement of PRL mRNA

As no species-specific immunoassay is available to quantify PRL in plasma for *N. pulcher*, the expression of PRL mRNA in the pituitary gland was determined as a measure of PRL production. In two related cichlid species (*Oreochromis mossambicus* and *O. niloticus*) there is a strong positive correlation between PRL mRNA levels in pituitary glands and PRL plasma levels (Auperin et al., '94; Shepherd et al., '99).

PRL transcript levels in pituitary glands collected from fish in nonreproducing aggregations were used as a reference to size matched family fish. Aggregation fish are not under the influence of changes in reproductive status or the presence of a brood, and they are not exposed to a strong dominance hierarchy or territoriality.

A total of eight independent family groups was sampled (breeder males: 58–87 mm standard length (SL), arithmetic mean = 68 mm; breeder females: 56.5–75 mm SL, mean = 62 mm; immature helpers: 26–35.5 mm SL, mean = 31.2 mm). For control samples, eight adult nonbreeding fish of each sex and ten immature fish from nonbreeding aggregations were chosen randomly (males: 59–77 mm SL, mean = 65.7 mm; females: 56–75 mm SL, mean = 68.8 mm; immature nonhelpers: 26–34 mm SL, mean = 30.2 mm). The sizes of experimental fish from family groups and respective controls from aggregations did not differ significantly from each other. Pituitary glands were collected the day after a clutch had been produced by an experimental group, as this period is most likely to reveal elevated PRL levels (see the Discussion). First, the breeders were separated from the breeding site by a sheet of clear acrylic glass so that they could not interfere with the clutch but had visual contact with the helpers in order to maintain the dominance hierarchy. The first helper showing direct brood care behavior was then selected for pituitary gland collection, along with the two breeders. Sampled fish were measured and weighed and rapidly decapitated. Their pituitaries were immediately collected and put in RNA later (Sigma, St. Louis, MO) and stored at −20°C until further processing.

Total RNA was extracted from individual pituitaries using Stratagene Absolutely RNA RT-PCR Microprep Kit (Stratagene Europe, Amsterdam, The Netherlands) following the manufacturer's protocol. cDNA synthesis was carried out with 30 μL extracted pituitary RNA in a 50 μL reaction mix containing: 0.4 mM dNTPs, 200 ng random hexamers (incubated at 65°C for 5 min, chilled on ice for 2 min), 13 μL RT-mastermix (30 mM dithiothreitol, 8 μL 5 × first strand buffer and 40 U RNase inhibitor) and 100 U reverse transcriptase (Superscript II, Invitrogen, San Diego, CA) added at room temperature. The reaction was incubated for 10 min at room temperature, then at 42°C for 50 min, followed by 70°C for 15 min to stop the reaction. The cDNA was stored at −20°C until analyzed.

Polymerase chain reaction (PCR) was carried out using 1 μL cDNA as template and PRL primers designed for *Salaria pavo* (spPRL_fw: 5′-TGC TCA GCC AGG AGC TGG ACT̄ C-3′ and spPRL_rv: 5′-CTG TCC TGG CCG AGG TCG TTG C-3′), which amplify a 450 bp product. The 25 μL reaction contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl (pH 8.8), 0.01% Tween-20, 2.5 mM $MgCl_2$, 0.12 mM dNTP, 10 pmol each of forward and reverse primers and 0.5 U Taq DNA polymerase (Euroclone, Pero, Italy). The thermocycling protocol was as follows: initial denaturing step (95°C, 2 min) followed by 35 cycles of denaturing (95°C, 45 sec), annealing (64°C, 45 sec) and extension (72°C, 30 sec) with a final elongation (72°C, 5 min). The PCR reaction products were run on a 1% agarose gel/tris borate ethylenediaminetetraacetic acid (TBE) containing ethidium bromide to ensure that a single amplification product was obtained. The resulting PCR product was cloned using the pGEM-T Easy Vector System (Promega, Madison, WI) and sequenced to ensure that the primers specifically amplified *N. pulcher* PRL. The sequence obtained (accessible in the NCBI GenBank at http://www.ncbi.nlm.nih.gov/entrez/sutils/girevhist.cgi, accession number DQ302760) was confirmed to code for PRL by a BLAST search (E value $<10^{-49}$) and used to design specific primers (PRPRLNP-130F: GCA CTT CAA GTA TCG GAG TCA GAT

T; PRPRLNP-130R: GTC CGA CCA GGC TTG GA) for real-time PCR (Custom TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA).

Semi-quantitative real-time PCR was carried out with *N. pulcher* pituitary cDNA as template and *N. pulcher* PRL specific TaqMan primers in a 25 μL reaction containing 12.5 μL 2 × ABI TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), 1.25 μL 20 × TaqMan Assay (*N. pulcher* specific primers), 6.25 μL distilled water and 5 μL cDNA. In order to evaluate the relative levels of expression of PRL, all pituitary samples were amplified in the same reaction and 18S ribosomal RNA (18S) was also determined (predeveloped assay, Applied Biosystems). Results for pituitary PRL mRNA were normalized for 18S, as 18S was shown to be a valid housekeeping gene in several studies (e.g. Bas et al., 2004). We assumed no major differences in the regulation of 18S in different phenotypes, but this has not yet been studied in *N. pulcher*. Real-time PCR was run on a GeneAmp 5700 Sequence Detection System (Applied Biosystems) using the standard thermal profile. All samples were run in duplicate and some samples without template were run simultaneously following the same protocol to serve as quality controls.

Analysis of PRL mRNA data

Data from two to four repeated measurements from the same samples were averaged, after confirming a high correlation between the repetitions (data not normally distributed, $n = 35$, Spearman's ρ correlation, PRL: $r_s = 0.942$, $P < 0.01$; 18S: $r_s = 0.873$, $P < 0.01$). PRL mRNA levels were normalized for 18S and calculated relative to one sample of a juvenile from the nonbreeding group, which showed a value in the middle of the range and which was set at 100. The presented data are therefore relative to this sample, allowing an easier comparison between the groups. The values were then square-root transformed to normalize the data. Samples that did not contain enough RNA (cycle numbers over 30 for both PRL and 18S) were removed from the analyses. All distributions were tested for outliers following Sokal and Rohlf ('81; p 413) and one outlier was detected and removed from the analyses. Eliminating two empty samples and one outlier, the total sample size was reduced from 32 to 29 adult fish. To calculate differences between treatments (social status) and sexes, a univariate analysis of variance was performed, separately for adults and juveniles. As PRL mRNA levels correlated significantly negatively with body mass (although normalized for 18S), body mass was incorporated as a covariate in the analyses to correct for body mass effects on PRL mRNA levels. Two-tailed statistical tests were performed with SPSS 11.0, with α set at 0.05.

Experimental manipulation of PRL levels

Seven male breeders and six immature helpers (sex unknown) of similar sizes to those used for PRL mRNA measurements were injected intraperitoneally with 0.06 IU/g body weight of commercially available ovine PRL (Sigma-Aldrich, St. Louis, MO) dissolved in teleost Ringer solution[2] at a concentration of 0.01 IU/μL. As the PRL had an activity of 31 IU/mg, 0.06 IU is equivalent to 1.94 μg. The dose was established after a pilot project with different PRL concentrations (0.03g–0.09 IU/g; $n = 36$). This range of doses has been shown previously to be effective in generating brood care behavior in several teleost fish species (Fiedler, '62; Blüm and Fiedler, '65; Blüm and Weber, '68; Molenda and Fiedler, '71). We tested the injected fish immediately, after 1, 2 and 3 days. The fish used were nonbreeding and tested toward experimentally presented eggs. We found only few positive reactions (brood care instead of egg feeding); these were all at the 0.06 dose and all at day 2 or 3 after injection.

A control group of seven male breeders and seven immature helpers (sex unknown) size matched to experimental fish was injected with teleost Ringer solution. Test fish and control fish were marked by fin-clipping if appropriate (Bergmüller et al., 2005; i.e. only helpers in families with several similar-sized helpers) and separated from the rest of the group by a sheet of clear acrylic glass during the experiment. All test and control families used were not breeding at the time of the experiment.

On the first day of the experiment, PRL or Ringer solution was injected in test and control fish, respectively. On each of 2 subsequent days, a test clutch was presented to test and control fish for a period of 30 min, respectively, and behavior was recorded for the entire period. Behaviors recorded were direct brood care (fanning and cleaning eggs), cannibalism (eating eggs) and

[2]Formulation of 1 L of freshwater teleost Ringer solution: distilled water 1 L, NaCl 6.49 g, KCl 0.186 g, MgCl 0.325 g, glucose 1.8 g, $CaCl_2$ 0.174 g.

digging sand from the shelter. The time between the injection and the recording of the behavioral reaction was based on data from previous studies (Fiedler, '62; Blüm and Fiedler, '65; Molenda and Fiedler, '71) and from our own pilot studies.

Test clutches were produced by 44 *N. pulcher* family groups not involved in the experiment and provided with clear polyethylene terephthalate (PET) linings in their shelters. Every morning each PET lining containing a clutch was removed and replaced with a new one. The collected eggs were kept in separate tanks at 24°C and well aerated with help of an air stone to avoid fungus growth. Test clutches of 1–2 days of age were presented to experimental fish by attaching the egg-bearing linings under flowerpot halves, which were placed in the test and control tanks in place of the breeding shelters at the start of each observation period. Eggs were counted before and after observations to determine the number of eggs eaten. Fungus-infected eggs were removed by the experimenter before observations.

Analysis of behavioral data collected during the PRL application experiment

Behavioral results from the PRL application experiment were summarized in categories of occurrence (brood care or no brood care, digging or no digging) and compared between family and control fish with the Fisher's exact test (two tailed).

RESULTS

PRL mRNA measurements of adults

PRL mRNA values were square-root transformed before analysis of covariance (ANCOVA) performance with body mass as covariate (adult family members: males, $n = 7$, females, $n = 8$; adult aggregation members: males, $n = 7$, females, $n = 7$).

Influence of sex: Overall, females showed significantly higher levels of PRL mRNA than males ($df = 1$; $F = 5.021$, $P = 0.035$). This difference occurred in both status groups ($df = 1$; for family members: $F = 4.945$, $P = 0.046$; for aggregation members: $F = 13.084$, $P = 0.004$; Fig. 1).

Influence of breeding status: Overall, nonreproducing aggregation fish showed significantly higher levels of PRL mRNA than family group members ($df = 1$; $F = 8.286$, $P = 0.009$). However, if analyzed separately for each sex, this difference was only significant in females ($df = 1$; for females: $F = 544$, $P = 0.0379$; for males: $F = 0.52$, $P = 0.487$; Fig. 1). The interaction between status and sex was not significant ($df = 1$; $F = 1.979$, $P = 0.173$).

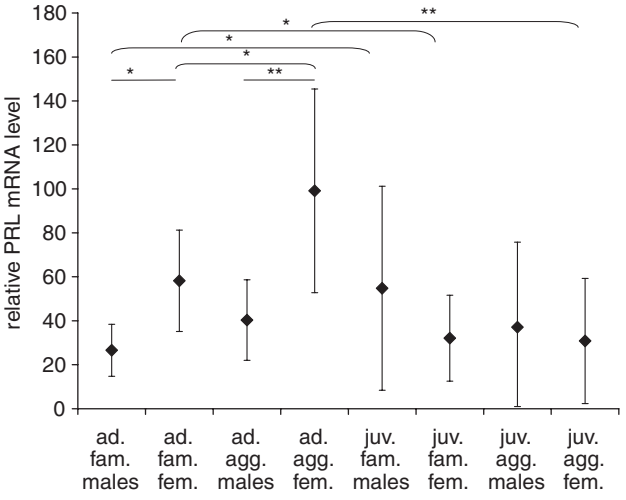
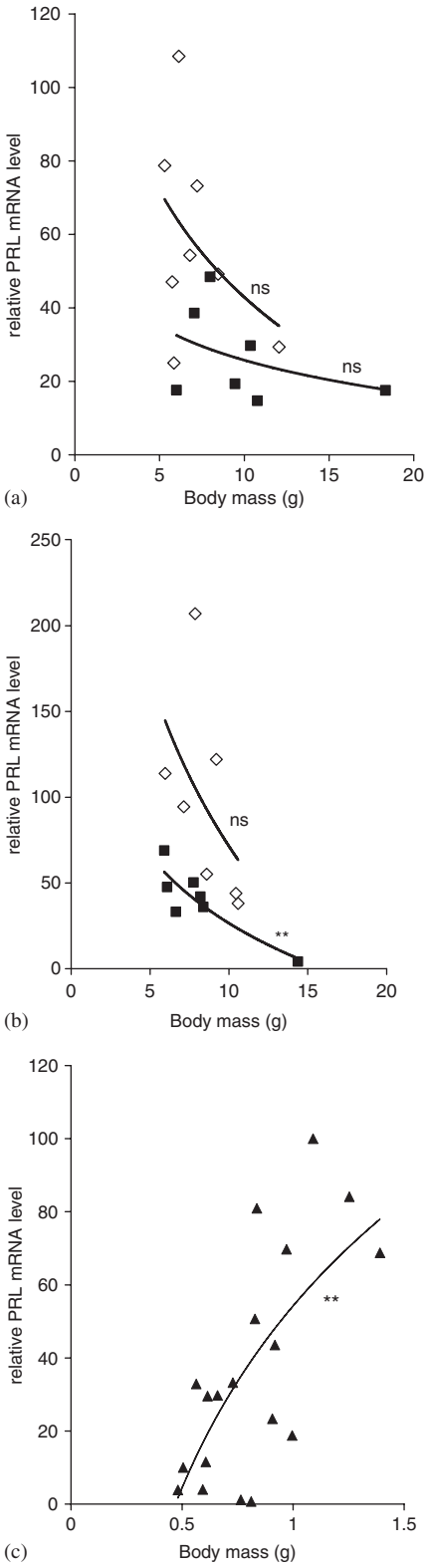


Fig. 1. Relative PRL mRNA levels separated by status and sex. Sample sizes: adult family males ($n = 7$), adult family females ($n = 8$), adult aggregation males ($n = 7$), adult aggregation females ($n = 7$), juvenile family males ($n = 3$), juvenile family females ($n = 6$), juvenile aggregation males ($n = 5$), juvenile aggregation females ($n = 5$). Hormone mRNA levels were square-root transformed before ANCOVA performance with body mass as covariate; mean ± SE are shown after back-transformation. For exact results and discussion see text. *Significance at $P < 0.05$, **significance at $P < 0.01$. PRL, prolactin; ANCOVA, analysis of covariance.

Influence of body mass: Overall, body mass showed a significant negative effect on PRL mRNA levels ($df = 1$; $F = 13.903$, $P = 0.001$). Furthermore, there was a significant interaction between status and mass suggesting different relationships between body mass and PRL mRNA levels in aggregation and family fish, respectively ($df = 1$; $F = 4.967$, $P = 0.036$). Indeed, the effect of body mass was significant only in aggregation members ($df = 1$; for family members: $F = 1.835$, $P = 0.2$; for aggregation members: $F = 10.563$, $P = 0.008$). If separated for sex within the status groups, mass had a significant effect on the PRL mRNA levels of aggregation males only ($df = 1$; aggregation males: $F = 30.46$, $P = 0.0027$; aggregation females: $F = 2.87$, $P = 0.15$; family males: $F = 0.92$, $P = 0.38$; family females: $F = 1.38$, $P = 0.284$; Fig. 2a,b).

There was no overall statistically significant effect of the interaction of sex and body mass on PRL mRNA levels ($df = 1$; $F = 1.195$, $P = 0.286$). The effect of body mass was significant in males ($df = 1$; $F = 7.12$, $P = 0.022$) and showed



a tendency in females (df = 1; $F = 3.74$, $P = 0.078$; Fig. 2a,b).

PRL mRNA measurements: juveniles

In juveniles (family members, $n = 9$; aggregation members, $n = 10$) only body mass had a significant effect on PRL mRNA levels, and in contrast to adults this effect was positive (df = 1; $F = 11.486$, $P = 0.004$; Fig. 2c). No differences were detected between family and aggregation members ($n = 19$; df = 1; $F = 0.684$, $P = 0.421$) and between males and females ($n = 19$; df = 1; $F = 0.882$, $P = 0.363$).

PRL mRNA measurements: comparison between adults and juveniles

Adults and juveniles within the same status and sex were compared by ANCOVA, including mass as covariate and the interaction between mass and age (see Fig. 1; ''age'' denotes if an animal is adult or juvenile).

In family males, adults had significantly lower PRL mRNA levels than juveniles ($n = 10$, 7 adults and 3 juveniles: df = 1; $F = 6.62$, $P = 0.0422$). The effect of mass was significant (df = 1; $F = 11.35$, $P = 0.0151$) as well as the interaction between mass and age (df = 1; $F = 12.12$, $P = 0.0131$).

In family females, adults had significantly higher PRL mRNA levels than juveniles ($n = 14$, 8 adults and 6 juveniles: df = 1; $F = 7.22$, $P = 0.0228$). There was no significant effect of mass (df = 1; $F = 3.24$, $P = 0.1022$) or of the interaction between mass and age (df = 1; $F = 3.90$, $P = 0.0764$).

In aggregation males, PRL mRNA levels tended to be higher in adults than in juveniles ($n = 12$, 7 adults and 5 juveniles: df = 1; $F = 5.15$, $P = 0.0530$). There was no significant effect of mass (df = 1; $F = 1.97$, $P = 0.1981$) or of the interaction between mass and age (df = 1; $F = 2.53$, $P = 0.1507$).

In aggregation females, adults had significantly higher PRL mRNA levels than juveniles ($n = 12$, 7

Fig. 2. Relative PRL mRNA levels in relation to body mass (g). For the statistical test of an association, PRL mRNA values were square-root transformed before ANCOVA performance with body mass as covariate. For exact results see text. (**a**) Adult family members, separated for females (white diamonds, $n = 8$) and males (black squares, $n = 7$), lines show logarithmic regression lines (above line for females, below line for males); the association is not significant for either sex; (**b**) adult aggregation members, symbols and lines as in (a) ($n = 7$ females and 7 males); the association is significant for males only; (**c**) juveniles (black triangles, $n = 19$), line as in (a); the association is significant. ns, not significant, **significance at $P < 0.01$. PRL, prolactin; ANCOVA, analysis of covariance.

adults and 5 juveniles: df = 1; $F = 12.18$, $P = 0.0082$). There were trends for an effect of mass (df = 1; $F = 4.26$, $P = 0.0730$) and an interaction effect between mass and age (df = 1; $F = 5.27$, $P = 0.0508$).

Experimental manipulation of PRL levels

Of the PRL-injected individuals (seven male breeders and six immature helpers), one male breeder and one helper showed brood care behavior. None of the fish in the control groups exhibited brood care (seven male breeders and seven immature helpers). No statistical difference was found between treated and control fish, both for male breeders (Fisher's exact test, $n = 14$, $P = 0.99$) and helpers (Fisher's exact test, $n = 13$, $P = 0.46$).

Six male breeders dug sand, whereas no helper did, which is a significant difference (Fisher's exact test, $n = 27$, $P = 0.015$). There was no difference in digging frequencies between treated male breeders and control male breeders (Fisher's exact test, $n = 14$, $P = 1$).

DISCUSSION

Many birds, mammals and fish show a clear positive relationship between PRL and brood care behavior (see above for references). In contrast, in this study we found lower pituitary PRL mRNA levels in breeding females compared with nonbreeding aggregation females. To our knowledge this is the first time that an inverse relationship between brood care behavior and PRL mRNA levels has been reported.

In our study PRL mRNA expression was measured in pituitaries collected on the day following spawning, which corresponds to the middle of the egg care stage in *N. pulcher*. Therefore, if PRL is important in the regulation of brood care in this species, it should be highest during this phase of the breeding cycle. In fact, in other fish and bird species PRL mRNA and plasma levels were elevated in breeders shortly after egg laying and in the middle of the brood care stage (*Aequidens portalegrensis*, Metuzals et al., '68; for birds, see Brown and Vleck, '98; Khan et al., 2001; Schoech et al., 2004; *O. niloticus*, Tacon et al., 2000). The observation of significantly lower levels of PRL mRNA in females engaging in brood care indicates that in *N. pulcher* maternal care may be associated with particularly low PRL levels. This is a riddle that we cannot resolve without further study.

In juveniles, no differences in pituitary PRL mRNA levels were detected between brood-caring helpers and aggregation members. Therefore, contrary to our expectations we found no indications for an effect of PRL on brood care behavior of alloparents in this species. We had expected an important role for PRL in the regulation of brood care behavior in sexually immature helpers, as in this group of fish other hormones such as sex steroids are unlikely to be involved. Also, PRL has been shown to be of particular importance for alloparental brood care in mammals and birds (for reviews, see Schradin and Anzenberger, '99; Ziegler, 2000). The lack of a difference in PRL mRNA levels between family juveniles and aggregation juveniles in *N. pulcher* might suggest that the differences found between breeding and nonbreeding females are more related to brood care behavior or the reproductive stage than to the social context of these females.

We found significantly higher levels of pituitary PRL mRNA in adult females than in adult males, both within nonreproductive aggregations and within reproductive family groups. Such significant sex difference in PRL mRNA expression patterns was also found in other species (e.g. in prespawning chum salmon, *Oncorhynchus keta*; Taniyama et al., '99). In juvenile *N. pulcher*, there was no sex difference in PRL mRNA levels, suggesting that the difference found in adults may be linked to sexual maturity. Indeed, a complex interplay between PRL and sexual steroids has been found in mammals, birds and fish (Brown, '85; Tacon et al., 2000; Khan et al., 2001; Cavaco et al., 2003; Schoech et al., 2004).

PRL mRNA levels decreased with increasing body mass in adult *N. pulcher* despite our calculation of mRNA levels relative to 18S levels, which is intriguing when considering the similar decline of steroid hormones with increasing mass found in an accompanying study (Bender et al., 2006). In the preceding study sex steroids and cortisol from fish holding water were measured and compared between breeder males and helper males of different sizes and showed also a negative correlation with body mass. Whether the decline in PRL and steroid hormones with increasing body mass is interrelated remains to be answered in future studies.

In contrast to the decline of PRL mRNA levels in adults with increasing body mass, in juveniles body mass and PRL mRNA levels correlated positively with each other. This finding agrees with results from several other fish species, where PRL levels were found to be lower in juveniles

than in adults (Pandolfi et al., 2001; Herrero-Turrión et al., 2003; Onuma et al., 2003). As this positive correlation of PRL and body mass in juveniles occurs also in species without brood care, it may reflect a common mechanism in fish development without major significance for brood care behavior.

The comparison between adult and juvenile fish of the same status and sex showed that in females, adult fish have significantly higher PRL mRNA levels than juveniles, both in families and in aggregations, whereas there was no systematic body mass effect. On the contrary, adult family males showed significantly lower PRL mRNA levels than juveniles, with a significant effect of mass and of the interaction between mass and age. Interestingly, the effect tended to be reversed in aggregation males.

The interpretation of these findings, however, is complicated by several factors. The experiment was designed to compare juvenile fish of either sex between families and aggregations, because it is hardly possible to determine the sex of juveniles reliably in living *N. pulcher*. Only after killing the juvenile fish in order to collect the pituitary glands it was possible to establish their sex. By chance they happened to be six family females, five aggregation females, five aggregation males and three family males. The resulting inequality of sample sizes between adult and juvenile fish and the partially very small juvenile samples reduce the significance of subgroup comparisons. More importantly, there are a number of potentially confounding factors associated with the condition of being dominant or subordinate, such as size, body mass, age, dominance, growth, sexual maturity level and reproductive status. In the comparisons between adults and juveniles we controlled for body mass, but not for other potential confounding variables. Furthermore, as we discussed above, PRL mRNA levels of juvenile and adult fish vary with body mass in opposite directions, decreasing in adults and increasing in juveniles with increasing body mass, with an interaction between body mass and status in adults, but not in juveniles. An effect of sex was found only in adults, but not in juveniles. In a subgroup analysis of adults and juveniles by status and sex it is not possible to account for interactions. For all these reasons, the comparisons between PRL mRNA level of adult and juvenile fish have to be interpreted with great caution.

Experimental application of PRL to male breeders and immature helpers did not alter their brood care behavior. Therefore, this experimental approach confirmed the conclusions from the correlative study of PRL mRNA levels that elevated levels of PRL are probably not directly involved in the regulation of brood care in this species. It is possible that the lack of an experimental effect might have been caused by the use of an inappropriate dose of ovine PRL. However, we think this is unlikely as in a pilot study we tested a range of concentrations between 0.03–0.09 IU/g PRL, with 0.06 IU/g showing the strongest effect (see the Materials and Methods section). Moreover, this range of doses of ovine PRL was found to effectively induce brood care behavior in other fish species (Fiedler, '62; Blüm and Fiedler, '65; Blüm and Weber, '68; Molenda and Fiedler, '71).

A number of other hormones such as progesterone (Brown, '85; Schoech et al., '91; Tacon et al., 2000) and growth hormone (Tacon et al., 2000) may be involved in the regulation of brood care behavior of parents and alloparents. In fact, priming with steroid hormones before PRL application led to brood care behavior in different fish species (see introduction). However, we did not combine our PRL treatment with steroid applications, as breeder and helper males in *N. pulcher* do not show fluctuating androgen levels during the breeding cycle (Bender et al., 2006). Female breeders were also found to resemble males in testosterone and brain gene expression profiles, despite their different roles in egg care (Aubin-Horth et al., 2007). Moreover, helpers show brood care behavior before sexual maturation, indicating that sex steroids are probably not important for regulation of their brood care behavior. A deficiency in brood care experience also cannot explain the lack of a response of brood care propensity to our PRL application, as all test fish were experienced in brood care.

As we pointed out in the introduction above, PRL is known to have over 200 different biological functions in vertebrates, in the context of growth, immune function and reproduction (Freeman et al., 2000). In fish it has an additional osmoregulatory function. It should be expected therefore that the application of PRL does not only affect brood care propensity, but also other behavioral and physiological mechanisms. Apart from digging, we did not record systematically other behaviors during our experiment. However, we did not observe major alterations in common behaviors such as aggressive and submissive displays or feeding. Similar experiments reported in the literature showed a positive effect of PRL on

brood care behavior despite the possible interactive effects of injected PRL.

Digging sand out of the breeding shelter is one of the behaviors related to brood care in *N. pulcher*. In our experiment male breeders showed significantly more digging behavior than helpers. Probably, this finding reflects a difference in status or age, but it was apparently not related to PRL as in male breeders digging was shown by the same number of treated and untreated animals.

In summary, our results suggest that elevated levels of PRL are not important for the regulation of brood care in *N. pulcher*. However, adult females showed higher PRL mRNA levels than adult males, and female breeders usually perform the most egg care behavior in this species (Taborsky, '84). The fact that nonbreeding females had significantly higher PRL mRNA levels than egg-caring females might point to a complex indirect role of PRL in the regulation of brood care of this species. One possibility is that a decrease in PRL mRNA might serve as a trigger for female brood care behavior in *N. pulcher*. Alternatively, a decrease in PRL mRNA could be connected to an elevation in plasma PRL. However, the results of our injection experiments in males and juveniles do not point in this direction.

The results from this study cast doubts on the hypothesized importance of PRL for parental and alloparental brood care in *N. pulcher*. This raises a number of questions about the proximate causes of parental and alloparental brood care behavior in *N. pulcher* and more generally in fish and in cooperatively breeding vertebrates, which will require further study.

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