Effects of confinement stress of variable duration on the growth and microincrement deposition in the otoliths of *Oreochromis niloticus* (Cichlidae)

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The effects of chronic confinement stress (1, 5 and 10 days) and of periodic blood sampling on somatic growth and the structure and growth of otoliths was studied in *Oreochromis niloticus*. During the study, the plasma concentrations of cortisol were measured at various times during the application of stress: they were significantly higher in confined fish than in control fish (mean ± s.d. 3.40 ± 0.47 vs. 1.26 ± 0.62 ng ml⁻¹, *P* < 0.05) up to 5 days after the start of a 10 day stress period. The somatic growth (standard length, *L*₅, and mass) was affected by the confinement and by the sampling (from 16.2 ± 1.07 to 14.64 ± 1.15 cm for *L*₅ and from 173.3 ± 33.14 to 110.5 ± 29.48 g for mass). But the confinement masked the effect of the sampling on somatic growth. Tetracycline was injected at the start of the experiment to mark the otoliths, and showed that the short and long duration confinements led to a clear check in the pattern of primary increments in the otoliths. The number of primary increments deposited during the resting periods that followed each period of confinement was always less than the number of days that these periods lasted. No relation was found between the duration of confinement and the structure of the resulting checks. These results suggest that there is a disruption in the laying down of primary increments during periods of confinement resulting in an underestimation of their number compared to the actual number of days of growth. These results call into question the use of otolith primary increments as a means of estimating the age of Nile tilapia that have experienced periods of stress.

Key words: checks; microstructures; *Oreochromis niloticus*; sagittae; stress.

INTRODUCTION

Nile tilapia *Oreochromis niloticus* (L.) (Cichlidae) is the main species of tilapia used in pisciculture throughout the world (Lazard, 1990; Gourene & Teugels, 1997) and accounts for the largest proportion of catches of cichlids in Africa (Fryer & Iles, 1972). To increase the production of animal protein, this species
has been introduced, or has been the subject of repeated stocking in many ponds and artificial reservoirs (Teugels et al., 1988; Lazard, 1990; Mikolasek et al., 2000), especially in regions where fish farming is difficult (Vallet, 1993; Baijot et al., 1994). The environmental conditions in these ponds are extremely variable and can lead to unfavourable living conditions, such as partial drying out causing confinement and poor water quality (e.g. long period of turbidity), that can last for variable lengths of time and put the fish under chronic stress. Monitoring the growth of fish in these rearing systems requires the use of biological markers.

Among the markers that can be employed, otolith microincrements are very widely used for estimating the age and growth of fishes since they were first described by Pannella (1971). In particular, the primary increments that are laid down on a daily basis are the most frequently used in such studies (Stevenson & Campana, 1992). They are formed by successive deposits of alternating layers rich in either inorganic or organic matter (Zhang, 1992). Their characteristics (width and contrast) can be affected by rearing conditions. For example food availability (Maillet & Checkley, 1990; Moksness et al., 1995; Paperno et al., 1997; Massou et al., 2002) decreased their contrast but did not affect their number in *O. niloticus* (Massou et al., 2002). Furthermore, food deprivation may result in smaller increments but not in the cessation of deposition if the individual has enough body energy reserves (Campana & Neilson, 1985). Temperature can also modify otolith growth (Gutierrez & Morales-Nin, 1986; Mosegaard et al., 1988; Volk et al., 1999; Oozeki & Watanabe, 2000; Reichert et al., 2000).

Difficulties in estimating age or backcalculating fish size from otoliths, however, have been encountered in various fish species because of the appearance of checks resulting from disruptions in otolith incremental deposition (Pannella, 1980; Campana & Neilson, 1985; Lagardère, 1989; Geffen & Nash, 1995). These checks interrupt the regularity of the primary increments (Pannella, 1980; Campana & Neilson, 1985; Morales-Nin, 1987a; Wright et al., 2002). There are many causes for the appearance of these checks. Some seem to be ontogenic as they appear at a fixed size irrespective of the age of the fish (Nishimura, 1993) whereas others are interpreted as being responses to particular physiological situations such as breeding or metamorphosis (Pannella, 1980; Campana & Neilson, 1985). But the main causes for these interruptions in growth are thought to be external stresses (Pannella, 1980; Campana & Neilson, 1985; Morales-Nin, 1987b).

Most studies on checks have dealt with their description (Gauldie et al., 1990; Zhang, 1992; Gauldie, 1993) and on the existence of a resorption phenomenon (mobilization of calcium) in otoliths during periods of stress (Campana, 1983; Ichii & Mugiya, 1983; Mugiya & Uchimura, 1989). As far as is known, there is no information establishing a relation between the type, intensity and duration of stress and the size of the check.

In the present study, the effects of chronic confinements of variable duration (between 1 and 10 days) on the structure, the number of primary increments and the growth of otoliths were investigated. Confinements are known to lead to stress in salmonids (Pickering et al., 1991) and in Nile tilapia (Auperin et al., 1997). The cortisol concentration, which is widely used as an indicator of stress (Mazeaud et al., 1977; Barton & Peter, 1982; Thomas, 1990), was measured in
O. niloticus subjected to chronic confinement. The somatic growth and condition factor were also used to assess the effects of the treatments on the overall physiological state of the fish. The experiments were conducted in controlled environments to minimize undesirable environmental disturbances.

MATERIALS AND METHODS

REARING CONDITIONS

Nile tilapias (‘Bouaké Strain’ from the Ivory Coast) were reared from August to October 1999 in an experimental closed-circuit installation supplied by dechlorinated tap water at the Institut National de l’Agronomie, Station Commune de Recherche en Ichtyophysiologie, Biodiversité et Environnement (INRA-SCRIBE), Rennes, France. This installation provided some control over water quality variables (mean ± range, temperature = 25.9 ± 0.4 °C; pH = 6.87 ± 0.36; dissolved oxygen = 7.28 ± 0.6 mg l⁻¹, ammonia <1 ppm; nitrite <2 ppm; nitrate <100 ppm). The photoperiod during the experiment was artificially maintained at 12L:12D. All the feeding, water sampling and cleaning operations were conducted with the minimum environmental disturbance to avoid undesirable stress. The food (BIOMAR trout pellets) was supplied at 3% of live mass throughout the light period. This quantity of food allowed the fish to grow well as determined in previous experiments (unpubl. data).

PREPARING THE FISH

Fish used were 264 days old at the start of the experiment. They all came from the eggs from a single female fertilized by the sperm of a single male. At the start of the experiment they were injected with a dose of tetracycline (50 mg kg⁻¹ live mass) and individually marked with magnetic PIT tags. The fish were divided into four uniform groups of 15 fish that had a mean ± s.d. standard length, \( L_S \), of 11.92 ± 0.71, 11.90 ± 0.49, 11.88 ± 0.51 and 11.85 ± 0.34 cm (ANOVA, \( P > 0.05 \)). Each group was placed in a 0.15 m³ tank (70 x 50 x 50 cm).

The experimental room was locked throughout the study and only people involved in the experimentation had access. Furthermore, care was taken to maintain all experimental groups in the same environmental conditions.

FISH TREATMENTS

The experiment started 15 days after placing fish in the tanks so that they could adapt to their new surroundings and recover from the stress of injecting the tetracycline and marking. The fish in the confined (C) and in the confined and sampled (CS) groups were subjected to three successive confinements of 1 day (on the 16th experimental day), 5 days (28th to 32nd experimental days) and 10 days (45th to 54th experimental days) interspersed with 12 or 13 days without confinement. The confinement was obtained by moving two grids together to increase the fish density in the tank from 8 (original density) to 133 kg m⁻³ (confinement density). The non-confined (NC) and non-confined and sampled (NCS) groups were reared without confinement (effective density of 8 kg m⁻³) throughout the experiment (71 days). For the CS and NCS groups, sampling consisted of capturing the fish in the tank and taking a small quantity of blood (c. 250 μl). Because confined fish did not eat (Auperin et al., 1997), all the fish groups were not fed during the confinement period and feeding was resumed after each confinement period. Thus the effect of confinement without the bias of the starvation induced by confinement was investigated. To assay cortisol during the 10 day confinement period, three fish were randomly captured in groups NCS and CS after 1, 5 and 10 days of confinement and blood samples were collected. The fish in groups C and NC were not disturbed in order to analyse the possible effect of blood sampling on the primary increments in otoliths.
Mortality was recorded only after the first confinement. This was one fish in each confined group and surprisingly two fish in the NCS group. These mortalities occurred within 3 days of the end of the first confinement period.

For welfare reasons, all the fish were fasted for 1 day before the end of the experiment. After sacrifice (high phenoxyethanol dose: 1 ml l\(^{-1}\)), they were weighed (\(M_T\), total mass in g), measured (\(L_S\) in cm) and then dissected to collect the otoliths (sagittae). These were cleaned in distilled water and stored in labelled tubes until preparation.

The radioimmunological assay of cortisol was conducted using 50 \(\mu\)l of plasma extracted with a 1:1 mixture of cyclohexane and ethyl acetate and redissolved in 300 \(\mu\)l of assay buffer according to Auperin \textit{et al.} (1997).

**PREPARATION AND EXAMINATION OF OTOLITHS**

The method of preparing thin sections for observation under transmitted light was derived from that described by Panfili & Tomáš (2001). It consisted of four main stages: (1) the \textit{sagitta} was embedded in a transparent polyester resin (Sody 33, ESCIL, France) that was allowed to harden for 24h; (2) a transverse section (2 mm thick) of the \textit{sagitta} containing the primordium was cut from the block of resin using a circular saw at slow speed (Isomet, BUEHLER); (3) this was then mounted on a microscope slide using a thermoplastic glue (Crystalbond 509). The section was ground using abrasive paper with grains of 25 \(\mu\)m and then 16 \(\mu\)m and polished with aqueous suspensions of 3 \(\mu\)m, 1 \(\mu\)m and 0.33 \(\mu\)m alumina pastes on a polishing cloth, until the primordium was reached; (4) the section was detached and turned over and the operation repeated on the other face until a thin section was obtained (10 to 40 \(\mu\)m thick). Only the left otoliths were used.

Preparations intended for observation by scanning electron microscopy (SEM) were made using a first stage that was similar to that of thin sections (\(n = 3\) for each group). As a second stage, the preparation was etched for 7 min with EDTA (ethylenediaminetetraacetic acid at pH 7.2) to create a micro surface relief. The surface was then sputtered with a thin layer of gold (10 nm) before being examined by SEM (Philips XL30).

The preparations (otoliths from the same fish as for SEM) were examined under transmitted light using a microscope system and camera that displayed the image on a video monitor. The final enlargement obtained varied from \(\times 400\) to \(\times 1000\). The primary increments and the checks formed after marking with tetracycline were counted along the internal ventral radius (IVR). The distinctiveness of the primary increments and the validity of their counts on this axis have been demonstrated in previous studies (Panfili & Tomáš, 2001; Massou \textit{et al.}, 2002). The ventral (VR), dorsal (DR), internal ventral (IVR) and internal dorsal radius (IDR) and their respective increase after marking with tetracycline \(VRA, DRA, IVRA\) and \(IDRA\) (Fig. 1) were measured using an image processing system dedicated to calcified structures (TNPC; Visilog, NOESIS, France).

**Fig. 1.** Growth axes measured on the transverse section of an otolith (sagitta) of \textit{Oreochromis niloticus}. \(DR\), length of dorsal radius; \(DRA\), increase in dorsal radius; \(VR\), length of ventral radius; \(VRA\), increase in ventral radius; \(IDR\), length of internal dorsal radius; \(IDRA\), increase in internal dorsal radius; \(IVR\), length of internal ventral radius; \(IVRA\), increase in internal ventral radius; tm, tetracycline mark.
DATA ANALYSIS

The somatic growth and that of the otolith were estimated from their specific growth $G_S$ calculated using the following general formula (Ricker, 1979): $G_S = 100 \left( \ln S_2 - \ln S_1 \right) t^{-1}$ where $S_1$ is the size recorded at the time of marking with tetracycline (either the $L_S$ of the fish or length of the otolith radius) and $S_2$ the size of the fish or the otolith at sacrifice, and $t$ is the time between marking and sacrifice.

The condition factor ($K$) was calculated using the formula $K = 100 M_T L_S^{-3}$.

The means of the data for the various groups were compared using ANOVA or, in the case of small samples, a Mann–Whitney $U$-test (STATISTICA software).

RESULTS

EFFECTS OF CONFINEMENT ON CORTISOL CONCENTRATIONS AND SOMATIC GROWTH

The plasma cortisol concentrations measured 1 day after the start of the third confinement period (Fig. 2) were significantly higher in the CS group than in the NCS group (mean $\pm$ s.d. $3.40 \pm 0.47$ v. $1.26 \pm 0.62$ ng ml$^{-1}$, $P < 0.05$) thus demonstrating the effectiveness of the stress treatment. Thereafter, the plasma cortisol concentration in the CS group decreased to reach values that were not significantly different from those of the NCS group at 5 and 10 days within the third confinement (mean $\pm$ s.d. $1.55 \pm 0.75$ and $1.80 \pm 0.68$ ng ml$^{-1}$ for CS v. $1.26 \pm 0.10$ and $0.77 \pm 0.21$ ng ml$^{-1}$ for NCS). The cortisol concentrations, after 24h, therefore reflected a progressive adaptation of the fish to chronic confinement.

On the whole, confinement and blood sampling reduced somatic growth since the mean $M_T$ and mean $L_S$ of the NCS, C and CS groups were significantly lower (ANOVA, $P < 0.05$) than those of the NC group (Table I). Furthermore, in the confined fish, the blood samplings seemed to lead to lowered growth in $L_T$ and $M_T$ (Table I). Significant effects of confinements, that were cumulative with those of the blood samplings, were also observed on $K$ (Table I).

![Fig. 2. Changes in mean + s.d. plasma cortisol concentrations in Oreochromis niloticus during the 10 day confinement period (third confinement period): after 24h (D1), 5 days (D5) and 10 days (D10). The values are for confined (CS, ■) and control non-confined (NCS, □) fish. *, Value significantly higher than that observed at the same time in the control non-confined group ($P < 0.05$).](image-url)
EFFECTS OF CONFINEMENT ON THE STRUCTURE AND GROWTH OF OTOLITHS

Characteristics of primary increments and checks

The primary increments were bipartite structures, composed of a zone translucent to light (L-zone) and an opaque zone (D-zone), interpreted as being daily deposits [Fig. 3(a)]. A check was a wider opaque zone with a greater contrast than the D-zones from the primary increments that surrounded it [Fig. 3(b), (c)]. The check therefore interrupted the regular appearance of the primary increments (Fig. 4) and was relative to the zones that surrounded it. Under SEM, the L-zone appeared as an raised zone (rich in crystalline material) whereas the D-zone appeared as a depression (rich in proteins). The mean ± s.d. width of primary increments was 0·73 ± 0·30 μm (n = 60 for three otoliths) and 1·28 ± 0·58 μm (n = 60 for three otoliths) for the D-zone and

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>( L_S ) (cm)</th>
<th>( M_T ) (g)</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>13</td>
<td>16·21 ± 1·07(^a)</td>
<td>173·31 ± 33·14(^a)</td>
<td>4·02 ± 0·28(^a)</td>
</tr>
<tr>
<td>NCS</td>
<td>12</td>
<td>15·20 ± 1·07(^b)</td>
<td>138·40 ± 23·66(^b)</td>
<td>3·87 ± 0·20(^{ab})</td>
</tr>
<tr>
<td>C</td>
<td>14</td>
<td>15·24 ± 0·72(^b)</td>
<td>131·75 ± 26·74(^b)</td>
<td>3·71 ± 0·27(^b)</td>
</tr>
<tr>
<td>CS</td>
<td>12</td>
<td>14·64 ± 1·15(^b)</td>
<td>110·50 ± 29·48(^b)</td>
<td>3·41 ± 0·32(^c)</td>
</tr>
</tbody>
</table>

TABLE I. Mean ± s.d. standard length, total mass and condition factor of the four groups of *Oreochromis niloticus* after 71 days of rearing including periods of confinement. NC, group reared without confinement; NCS, group reared without confinement but subjected to blood sampling; C, group subjected to confinements of 1 day (16th experimental day), 5 days (28th to 32nd experimental days) and 10 days (45th to 54th experimental days) without blood sampling; CS, as for C but with blood sampling. \( n \), group size. The same superscript letter given after the means of a column signifies that the values do not differ significantly between groups (\( P > 0·05 \)).

Fig. 3. Internal ventral margins of transverse sections of *Oreochromis niloticus* sagittae observed by optical microscopy: (a) primary increments with translucent L-zones and opaque D-zones, (b) detailed view of a main check mark (>) and (c) detailed view of a secondary check (>}). Scale bar = 20 μm.
FIG. 4. Internal ventral margins of transverse sections of Oreochromis niloticus sagittae observed by optical microscopy: (a) non-confined control (scale bar = 50 μm) and (b) fish subjected to three confinements of 1 day (C1), 5 days (C5) and 10 days (C10) applied respectively from the 16th, 28th and 48th days (total experiment duration = 71 days); from bottom to the top, the main C1 check and then those of C5 and C10; ▲, the first secondary check of C5 and that of C10 (scale bar = 25 μm). ➝, Check caused by tetracycline marking.
L-zone respectively. Checks formed deeper and much more clearly pronounced depressions than the surrounding D-zones (Fig. 5). The mean ± s.d. dimensions of main and secondary checks were 14.28 ± 7.71 μm (n = 8 for four otoliths) and 4.09 ± 0.97 μm (n = 5 for three otoliths) respectively.

The examination under transmitted light demonstrated the existence of a main check close to the tetracycline mark that was observed on all otoliths (Fig. 4). This check was followed by a portion of primary increments that contrasted less than in the other regions of the marginal zone that was studied, and sometimes by one or two less pronounced secondary checks very close to the main one.

Each period of confinement was characterized by a main check followed by a zone where primary increments were laid down [Fig. 4(b)]. More or less pronounced secondary checks were observed within these primary increments but it could not be determined whether or not they were regular [Fig. 4(b)]. The checks caused by the first confinement (1 day) were on average more contrasted and wider than those related to the two subsequent confinements (5 and 10 days). But in the manipulated groups, whether or not confined, no check was noted that could be temporally related to the handling that took place during blood samplings (10 days confinement).

The SEM (three preparations from each group) did not reveal any thinner primary increments that were not observable with the compound microscope.

![Image](image_url)

**Fig. 5.** Transverse section (ventral sulcal margin) of an *Oreochromis niloticus sagitta* observed by scanning electron microscope [otolith came from the same fish as that used in Fig. 4(b)]. Fish subjected to three confinements of 1 day (C1), 5 days (C5) and 10 days (C10) applied respectively from the 16th, 28th and 48th days (total duration of experiment = 71 days); from bottom to the top: ➣, the main check caused by the tetracycline marking; ➞, the main C1 check and then those of C5 and C10; ▼, the first secondary check of C5 and that of C10 (scale bar = 50 μm).
with a lower resolution. It was found that the area containing the primary increment deposits that occurred after the marking with tetracycline had only a slight contrast and were better able to withstand the etching by EDTA (Fig. 5). But this resistance was not observed in the area separating the checks related to confinement. Nevertheless the acid etching and the SEM images did reveal the main checks in the otoliths without ambiguity.

Each period of stress (injection of tetracycline and confinements), excluding the blood samplings, therefore resulted in a main check separated by distinct microincrements and secondary checks. These results thus confirm the previous observations made with the light microscope.

Number of microincrements and age estimation

At the end of the experiment, the number of primary increments counted, using the light microscope, on the internal ventral radius (IVR) showed a significant underestimate in the number of days elapsed after the marking with tetracycline in all groups, but this was more pronounced for the groups NCS, C and CS (Table II). The results therefore show a significant effect of the treatments (confinements and blood samplings) in reducing the daily deposition of the primary increments. This underestimate of the true age seemed to increase with the severity of the stress endured by the fish (Table II).

By analysing the otoliths of confined fish, in which the periods of stress could be located (check to check numbering of primary increments), it was found that the number of primary increments was always less than the duration of the corresponding period (Table III). In contrast this number was generally similar to the duration of the period of non-confinement. Therefore the effect of confinement increased with its length. The difference between the number of primary increments and the duration of the period comprising one confinement and the following non-confinement period increased with the duration of the confinement: c. 2, 4 and 8 days (difference = −15, −29 and −49%) respectively for C1C5, C5C10 and C10M (Table III and Fig. 6). Furthermore, the marking with tetracycline also had a marked effect on the laying down of primary

**Table II.** Mean ± s.d. number of primary increments counted along the internal ventral radius on transverse sections of the sagittae in the four groups of *Oreochromis niloticus* after 71 days of rearing including periods of confinement. NC, group reared without confinement; NCS, group reared without confinement but subjected to blood samplings; C, group subjected to confinements of 1 day (16th experimental day), 5 days (28th to 32nd experimental days) and 10 days (45th to 54th experimental days) without blood sampling; CS, as for C but with blood sampling. n, group size. The same superscript letter given after the means signifies that the values do not differ significantly between groups (P > 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Number of days after marking</th>
<th>Number of primary increments</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>10</td>
<td>71</td>
<td>66.17 ± 0.86^a</td>
</tr>
<tr>
<td>NCS</td>
<td>9</td>
<td>71</td>
<td>59.50 ± 0.71^b</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>71</td>
<td>47.40 ± 0.41^c</td>
</tr>
<tr>
<td>CS</td>
<td>9</td>
<td>71</td>
<td>46.75 ± 1.00^c</td>
</tr>
</tbody>
</table>
increments since the difference that corresponded to this event was c. 3 days before the first confinement (Fig. 6). All the stresses inflicted on the fish therefore disturbed the deposition of primary increments on the *sagittae*.

**Otolith growth**

The NC group had the highest specific otolith growth rate (\(G_S\)) \((P < 0.05)\) (Table IV) on three growth axes of the *sagitta* (\(VR\), \(DR\) and \(IDR\)). In terms of the transverse axes, the internal ventral radius (\(IVR\)) was insensitive to blood sampling and confinement. There was no significant difference in the otolith growth along any of the axes between the NCS, C and CS fish (ANOVA, \(P > 0.05)\).

**Table III.** Mean ± s.d. number of primary increments deposited on the internal ventral radius of *sagittae* of *Oreochromis niloticus* during the three successive non-confinement periods. TC1, C1C5, C5C10, C10M correspond respectively to the periods between the start of rearing (marking with tetracycline, T) and the start of C1, between the start of C1 and the start of C5, between start of C5 and the start of C10 and between start of C10 and the end of the experiment (M, otolith margin). C, confined group; CS, confined and sampled group \((n = 3)\). The same superscript letter given after the means of a column signifies that the values do not differ significantly between groups \((P > 0.05)\).

<table>
<thead>
<tr>
<th>Period</th>
<th>TC1</th>
<th>C1C5</th>
<th>C5C10</th>
<th>C10M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (days) ((\text{Confinement} + \text{non-confinement}))</td>
<td>15 ((1 + 12))</td>
<td>13 ((5 + 13))</td>
<td>18 ((10 + 15))</td>
<td>25 ((15 + 10))</td>
</tr>
<tr>
<td>C</td>
<td>11.33 ± 0.47a</td>
<td>11.00 ± 1.41a</td>
<td>14.33 ± 1.25a</td>
<td>17.33 ± 0.94a</td>
</tr>
<tr>
<td>CS</td>
<td>12.00 ± 1.41a</td>
<td>11.67 ± 1.70a</td>
<td>13.67 ± 1.25a</td>
<td>17.67 ± 0.94a</td>
</tr>
</tbody>
</table>

**Fig. 6.** Difference between the real number of days of rearing and the number of primary increments observed in the otoliths at each stage of confinement of *Oreochromis niloticus* for the groups without (group C, □; \(n = 3\) fish for each period) or with blood sampling (group CS, ■; \(n = 3\) fish for each period).
The distances between the two successive checks are given in Table V. The main check probably indicated the start of one confinement and the following secondary checks represented the end of the same confinement. Measurements are expressed as a percentage of total growth. As with the counts of microincrements, this analysis was only conducted on the confined fish, which were the only ones with checks that could be easily attributed to the treatment applied to the fish.

The relative otolith growth during any confinement period varied from 7.61 to 11.11% of the total internal ventral radius growth. These relative growths were not therefore proportional to the duration of the corresponding confinement. Furthermore, during the periods of 12 to 13 days separating the periods of stress, the percentage relative growth in size of the otoliths was not constant. For example, the distance C1C5 (mean ± s.d. 9.68 ± 3.02 for C and 8.32 ± 0.6% for CS) was lower (but not significantly) than the distance C5C10 (12.96 ± 2.36 for C and 15.35 ± 4.36% for CS).

**DISCUSSION**

**EFFECT OF TREATMENTS ON PLASMA CORTISOL AND SOMATIC GROWTH**

Following previous works of Mazeaud *et al.* (1977), Barton & Peter (1982) and Thomas (1990), the plasma cortisol concentrations were used in the present experiment for monitoring the severity of stress in the fish. Confinement (C10), but not starvation, evidently led to stress since the cortisol concentration measured after only 1 day of confinement was significantly higher than that of non-confined fish. The cortisol concentration measured in the study was, however, lower than that reported after 1 day of confinement in *O. niloticus* by Auperin *et al.* (1997) and can be explained by the fact the fish in the present study had been previously exposed to two periods of confinement (C1 and C5).
Table V. Relative growth (%) of the internal ventral radius (mean ± s.d.) of *Oreochromis niloticus* during three successive confinements of 1 day (C1), 5 days (C5) and 10 days (C10) applied respectively from the 16th, 28th and 45th experimental days of rearing (total duration of rearing = 71 days). C1, C5 and C10 are respectively the portions of the otolith deposited between the check that in principle corresponds to the C1, C5 and C10 periods of confinement; TC1, C1C5, C5C10 and C10M are respectively the portions deposited during the period of non-confinement between the marking with tetracycline (T) and C1, between C1 and C5, between C5 and C10 and between C10 and the margin of the otolith (M). C, confined group (n = 7); CS, confined group subjected to blood sampling (n = 6). The same superscript letter given after the means of a column signifies that the values do not differ significantly between groups (P > 0.05)

<table>
<thead>
<tr>
<th>Group</th>
<th>TC1</th>
<th>C1</th>
<th>C1C5</th>
<th>C5</th>
<th>C5C10</th>
<th>C10</th>
<th>C10M</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>25.14 ± 2.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.11 ± 2.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.68 ± 3.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.15 ± 1.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.96 ± 2.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.10 ± 1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.85 ± 1.41&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CS</td>
<td>23.56 ± 3.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.61 ± 1.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.32 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.62 ± 3.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.35 ± 4.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.71 ± 2.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.80 ± 4.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>
In fact, it has been shown in other fish species that individuals that have been subjected to repeated stress, have progressively diminished responses with time (Pickering & Pottinger, 1985; Barton & Schreck, 1987; Barton et al., 1987).

During the 10 day period of confinement, the plasma cortisol concentrations also decreased in the confined fish, to become not significantly different from the control fish after only 5 days. The low levels (c. 1 ng ml\(^{-1}\)) (Auperin et al., 1997) measured in control fish and in confined fish after 5 days, once more suggests that the fish were well adapted to their environment and that rearing conditions were good.

The mass and \(L_s\) measured at the end of the experiment for confined fish and fish handled for blood sampling, indicated a reduced growth for the fish of these groups. This is in agreement with the adverse effects on somatic growth of various stressful factors that have already been described in the literature (Barton & Peter, 1982; Thomas, 1990; Toguyeni, 1996). The present results also suggest that even brief stressful events such as blood sampling can have a lasting effect on growth, since there was a significant difference in growth between the groups NC and NCS. This persistence can be explained by the secondary metabolic effects of stress (Mazeaud et al., 1977). The cumulative effects of different types of stress are particularly well reflected by \(K\).

Because of the complex and time-consuming experimental design, only one tank per treatment was used but a combination of treatments (e.g. C v. CS and C v. NC) was employed. Several tanks are often used in growth experiments to test the effect of uncontrolled stress. In the present experiment, this kind of problem was not apparent because the control group had the best growth, size and condition. This result confirmed that this group had not been disturbed during the experiment, which validates these results.

**EFFECT OF CONFINEMENT STRESS ON OTOLITHS**

Growth along the various otolith axes is affected in several ways by stress. For example, the sampling and the associated handling decreased growth along the longitudinal axes of otoliths in non-confined Nile tilapia but had no effect on those of confined fish. The longitudinal axes (\(VR\) and \(DR\)) showed differences in growth between groups similar to those observed for growth in \(M_T\) and \(L_s\).

As for the transverse axes, the handling had a significant effect on the growth of the internal dorsal radius (\(IDR\)) only in the non-confined fish. This relative stability in growth along certain axes of the *sagitta* has also been shown in previous studies (Massou et al., 2002) and can be explained by the low coupling between otolith growth and somatic growth. Although the regular arrangement of the primary increments justifies their use for age estimation of fishes not subjected to stress (Panfili & Tomás, 2001), the use of the width of these increments does not seem to be suitable for estimating the somatic growth of this species.

Some of the treatments applied (injection of tetracycline and confinements) led to the appearance of checks. Blood sampling in confined fish and starvation during the period of confinement, however, did not (no check in non-confined
fish). Checks were clearly distinct on the internal margin of the sulcus as has been found in previous studies on the effects of chronic food restrictions (15 to 30 days) (Massou et al., 2002). The analysis of checks caused by the different treatments showed that there was a main check followed by an area of regular growth in which other less pronounced checks occurred.

In *O. niloticus*, the check related to the marking with tetracycline corresponded to the start of this operation (Panfili & Tomâs, 2001; Massou et al., 2002). The trauma caused by this marking disturbed the regular deposition of primary increments for 3 to 4 days before the restart of regular deposition (Panfili & Tomâs, 2001; Massou et al., 2002). In the present experiment, it was therefore likely that the main check marked the start of the application of confinements, for which there has been no previous study of their specific effects. The formation of secondary checks then occurred during and after the end of confinement.

The mechanism explaining the formation of secondary checks at the removal of stress is still unknown. Similar results have not apparently been published before. Only Zhang & Runham (1992) have reported the formation of a second check in *O. niloticus* during the passage from sub-optimal to ideal conditions (increase in temperature and end of fasting). These authors formulated the hypothesis that this change in conditions could be stressful for fish. Unfortunately, in this study, there was no information available on the number of primary increments deposited before the appearance of the second check. Furthermore, the second check mentioned by Zhang & Runham (1992) was not observed by Massou et al. (2002) during food restriction (1% of live mass) that was applied to juvenile *O. niloticus*. It is also possible that these secondary checks are due to a modification of metabolic processes in confined fish. For example, calcium metabolism can be modified by stress (Campana, 1983; Ichii & Mugiya, 1983; Mugiya & Uchimura, 1989).

The three confinements led to age underestimates with significant differences between the number of primary increments and the number of days between each confinement of c. 2, 4 and 8 days respectively. The possible inaccuracy of observations by optical microscopy due to resolution limits has previously been mentioned (Morales-Nin, 1988) but SEM was unable to reveal thinner primary increments, thus confirming the underestimation of the age. It is interesting to note that this difference was less than the duration of confinement (except in the case of the 1 day confinement), which confirms the fact that primary increments were definitely laid down if confinement lasted for several days. But there was no relation between the duration of confinement and the structure of the resulting checks. The small difference between the number of primary increments (in NC fish) and the number of days in the experiment could also be a result of the initial marking with tetracycline.

Further studies, with for example a double marking with tetracycline at the start and end of confinement, are however needed to show whether primary increments are formed during chronic stress. Such studies, possibly combined with studies in the natural environment, could confirm whether the marks observed during this study are specific or not. The results of this experiment also showed that stress had an adverse effect on otolith growth (longitudinal axis) in similar proportions to that on somatic growth. The appearance of
checks related to stress, without any relation to its duration, makes impossible to estimate the age of fish that have experienced periods of difficult living conditions.

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