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Validating and corroborating the deposition of two annual growth zones in asteriscus otoliths of common carp *Cyprinus carpio* from South Africa's largest impoundment

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A total of 816 common carp *Cyprinus carpio* asteriscus otolith pairs were collected from Lake Gariep, South Africa. Otoliths were interpreted whole, submerged in methyl salicylate and viewed under transmitted light. The precision of growth zone counts of the primary reader was estimated at 5·54 and 7·03% using the average per cent error method and the coefficient of variation, respectively. Age-bias plots indicated no systematic bias between the primary reader and the three secondary readers for up to nine growth zones (95% of the sample). Growth zone deposition rate was validated using a mark–recapture experiment of chemically tagged *C. carpio* ($n = 21$) conducted in a large earthen pond under ambient conditions in the vicinity of Lake Gariep. The validation results were corroborated for the wild population by edge analysis and a length-based age-structured model. All three methods suggest that growth zone formation occurred biannually, exemplifying the importance of age validation as a prerequisite for understanding the life history of *C. carpio*.

Key words: biannual; edge analysis; Lake Gariep; length-frequency analysis; oxytetracycline.

INTRODUCTION

Common carp *Cyprinus carpio* L. is a species of worldwide importance and concern. Native to Eastern Europe and Central Asia, *C. carpio* is the most widely translocated freshwater fish species with introductions reported from >120 countries (Casal, 2006) and is established in all continents except Antarctica (Koehn, 2004). It is perceived to be an aggressive invader with the potential to rapidly establish in newly invaded ecosystems as its life-history characteristics include fast growth, early maturity, high reproductive capacity and a short population doubling time (Brown & Walker, 2004; Koehn, 2004; Zambrano *et al*., 2006; Britton *et al*., 2007).

Accurate ageing is crucial for determining growth, age at sexual maturity and mortality (Campana & Thorrold, 2000), life-history traits regarded as directly related to the fitness of a species in a given environment (Roff, 1984). Structures that have been used for ageing *C. carpio* include scales, opercula bones, vertebrae, dorsal

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spines, pectoral fin rays and otoliths (Vilizzi & Walker, 1999; Britton *et al*., 2007; Jackson & Quist, 2007; Phelps *et al*., 2007). These studies have varied in their success, and there is a lack of consensus about the most appropriate structure. In recent studies, dorsal spines and asteriscus otoliths have been considered the most reliable in terms of accuracy and precision (Vilizzi & Walker, 1999; Brown *et al*., 2004; Jackson & Quist, 2007; Phelps *et al*., 2007).

Although *C. carpio* is one of the better studied cyprinids, detailed ageing studies based on formal validation of growth zone deposition are rare, and to date, asteriscus otoliths represent the only hard structure that has been validated using fluorochrome marking (Brown *et al*., 2004). Only one study applied marginal increment and edge analysis to determine growth increment formation in scales, opercular bones and asteriscus otoliths (Vilizzi & Walker, 1999). Both studies were conducted on *C. carpio* populations of the Murray-Darling Basin, Australia, and revealed evidence that one growth zone was deposited annually.

In Africa, published literature on ageing *C. carpio* is virtually non-existent. In a recent study, Britton *et al*. (2007) collected scales to derive age estimates for *C. carpio* from Lake Naivasha, Kenya, but were confronted with a lack of consistency even within the same specimen, and in some cases, a complete absence of growth checks. In two South African studies, scales (Hamman, 1981) and vertebrae (Cochrane, 1985) were used for determining age and growth.

To contribute to the knowledge on accurate ageing of one of the most prominent invasive and widely distributed freshwater fishes, this study aimed to validate the rate of growth zone deposition in asteriscus otoliths of *C. carpio* from Lake Gariep, South Africa. The validation approach included a mark–recapture experiment of chemically tagged fish that was conducted in large earthen ponds under ambient conditions in the vicinity of Lake Gariep. The validation results were then corroborated for the wild population using edge analysis and by applying a length-based age-structured model. For both methods, the null hypotheses that growth zone deposition is annual and that growth zone deposition occurs biannually were tested. The findings of this study represent the first validation of growth increments in otoliths of an African *C. carpio* population.

MATERIALS AND METHODS

STUDY AREA

Lake Gariep (30° 38′ S; 25° 46′ E; 1250 m above mean sea level), a hydroelectric impoundment on the upper Orange River, is situated between the Eastern Cape, Northern Cape and Free State provinces (Fig. 1). It is South Africa's largest inland water body with a surface area of *c*. 360 km^2 and an average depth of 16·3 m at full capacity (Hamman, 1981).

Lake Gariep is characterized as an oligo-mesotrophic impoundment that is turbid due to high levels of silt that are carried-in by the Orange River (Keulder, 1979). Over the study period mean Secchi disc depth was 15 cm (range: 5–34 cm). The climate in this region is considered semi-arid and rainfall is seasonal with most of the *c*. 400 mm of annual precipitation falling in summer (Keulder, 1979). Water level fluctuates considerably and is a function of the inflow of the Orange River, usually during spring or summer, and water release for power and water supply demands. The *c*. 400 km long shoreline is primarily comprised of extensive, gradually sloping shores, which are largely devoid of vegetation (Hamman, 1981). The remaining shoreline (10%) is steep and rocky (Cambray *et al*., 1978).

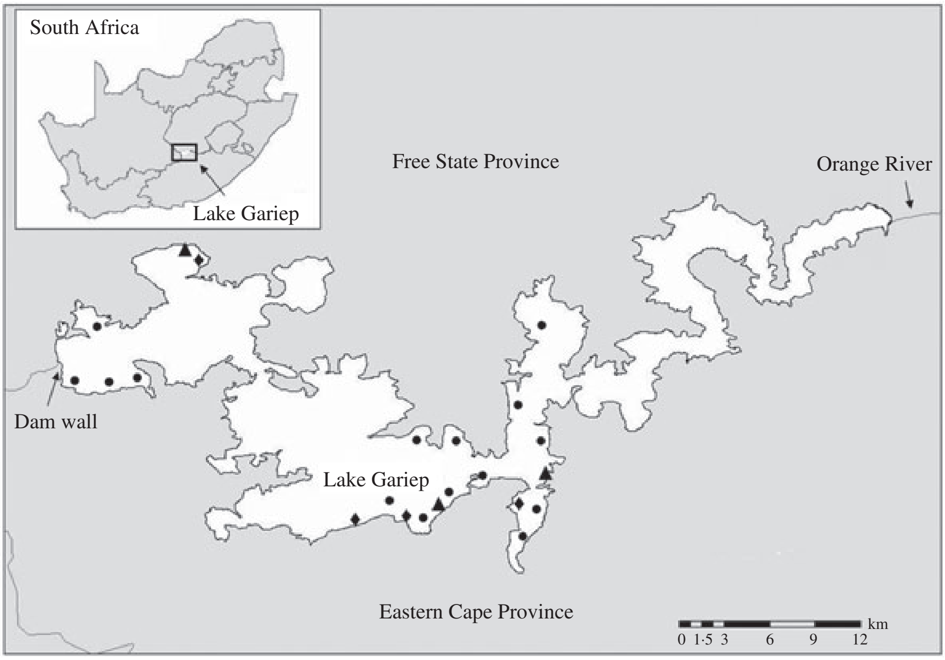

FIG. 1. Map of Lake Gariep, South Africa, showing the location of sampled angling competitions (▲), gillnet sites (●) and seine sampling sites (◆).

During spring and summer, the exposed drawdown zone is colonized by a short-lived plant community (Cambray *et al.*, 1978). Annual average surface water temperature between May 2007 and May 2008 was 15·9° C. The mean surface water temperature was 21·4° C (range: 16·6–26° C) in summer and 10·2° C (8·7–11·3° C) in winter.

SAMPLING

Field sampling was conducted from November 2006 until May 2008. The sampling protocol was dynamically adjusted according to feasibility, constraints, sampling occasion and sample size demands for later biological analysis. The largest samples for biological analysis were donated by anglers during shore angling competition events held in November 2006, January 2007, April 2007 and October 2007. Additional samples were obtained during bimonthly gillnet surveys (multifilament: 44, 60, 75, 100, 144 mm stretched mesh-size) conducted between March 2007 and May 2008, from seining experiments (April 2007, January 2008 and May 2008) and from occasional purchases from shore anglers. All fish were measured by the research team to the nearest mm for fork length, L_F. Sub-samples of measured fish were selected at random, sacrificed and the asteriscus otoliths were removed and stored dry in 1·5 ml Eppendorf tubes for later analysis.

OTOLITH INTERPRETATION

As different ageing procedures have been used for different populations of the same species (Jackson & Quist, 2007; Richardson *et al.*, 2009), various approaches were assessed to determine the most suitable method for interpreting growth. These approaches included: (1) using the ventral lobe of 0·4 mm thick otolith sections, cut transversely through the nucleus and viewed under transmitted light (Brown *et al.*, 2004); (2) using whole otoliths submerged in water and viewed under reflected light with a dark background (Vilizzi & Walker, 1999) and

FIG. 2. Photomicrograph showing alternating translucent (light) and opaque (dark) zones of a whole asteriscus otolith of *Cyprinus carpio* that was submerged in methyl salicylate and viewed under transmitted light. ○, Opaque zones along the antero-dorsal ageing transect.

(3) examining whole otoliths submerged in methyl salicylate and viewed under transmitted light using varying magnifications (×10 to ×40). Highest confidence in the optical interpretation of growth zones was achieved using the third method, which has also been suggested for determining age in asteriscus otoliths of other cyprinid species in South Africa (Potts *et al*., 2006; Weyl *et al*., 2009). This method was therefore accepted as the standard protocol for this study.

Growth zones were visible as alternating translucent (light) and opaque (dark) zones (Fig. 2). The number of growth zones were determined by counting the numbers of opaque zones along an antero-dorsal ageing transect situated on the otolith rostrum (Fig. 2). All otoliths ($n = 816$) were read at least twice by the first author, at random, at least 2 weeks apart and without knowledge of the date of capture or L_F of the fish. If the two readings were identical, the count of growth zones was accepted. If the two readings differed, a third reading was conducted. If this resulted in two identical readings, the counts were accepted. If the three readings differed at most by two growth zones (*e.g.* 2, 3 and 4), the median estimate was accepted, otherwise the otolith was rejected.

ACCURACY AND PRECISION

The precision of growth zone counts of the primary reader was assessed using the average per cent error (APE) method (Beamish & Fournier, 1981) and by estimating the coefficient of variation (C.V.) suggested by Chang (1982). An experiment was conducted to account for systematic biases that may affect the accuracy of growth zone counts. For this purpose, 100 otoliths were randomly selected from the data set and read once by three secondary readers. The results were compared to the accepted counts that were conducted by the primary reader. All secondary readers were experienced in the interpretation of otoliths but unfamiliar with *C. carpio* otoliths. Each reading was therefore conducted without any prior knowledge of criteria applied by the primary reader, date of collection or size of the fish. Age-bias plots (Campana *et al*., 1995) were used to assess the level of bias between primary and secondary readers. The assumption of a 1:1 relationship was assessed by linear regression t-tests to test if the slope and the intercept were significantly different from one and zero, respectively (Roff, 1984; Weyl & Booth, 2008).

FLUOROCHROME MARKING EXPERIMENT

Oxytetracycline hydrochloride (OTC) was used to validate the frequency of otolith growth zone deposition. OTC rapidly chelates in otoliths and produces a fluorescent mark when viewed with ultraviolet light (Campana, 1999). Mark-recapture of OTC-marked wild fish was suggested to be one of the most suitable methods available to validate growth zone periodicity (Campana, 2001). The vast surface area of the dam and the limitation of subsistence and recreational angling to <20% of the shoreline (Ellender *et al*., 2009) precluded the use of mark-recapture of OTC-marked wild fish as a suitable method because recapture probabilities were low. OTC-marked wild-caught *C. carpio* from Lake Gariep were, therefore, released into a large earthen pond under ambient conditions situated at the Lake Gariep State Fish Hatchery that is located 3·2 km below the dam wall on the banks of the Orange River (30° 37′ 584″ S; 25° 28′ 229″ E). The pond was 50 m long × 25 m wide × 1·5 m deep and stocked with 21 wild *C. carpio* ranging from 295 to 460 mm L_F caught by angling during March 2007. On capture, fish were placed in a water-filled canvas bag and carried to an oxygenated holding tank that contained 0·2 ml l^{-1} of the anaesthetic 2-phenoxyethanol (Sigma-Aldrich Chemicals; www.sigmaaldrich.com) to minimize postcapture stress until transported to the pond. On site, fish were injected with 60 mg kg^{-1} fish mass OTC solution (HiTet 120, Bayer; www.bayer.com) prior to release into the pond. As a control and to monitor the growth rates during the course of the experiment, all fish were tagged with T-bar anchor tags (TBA-2, Hallprint; www.hallprint.com). Stocking density was kept below one fish per 90 m^3, and there was no supplementary feeding. Fish were recaptured after 14 months at liberty (in May 2008), sacrificed, measured (L_F) to nearest mm, weighed to the nearest g, sex was determined macroscopically and the asteriscus otoliths removed and stored dry in 1·5 ml Eppendorf tubes. Otoliths were stored in the dark to prevent the degrading effect that UV light has on OTC.

The results from the only other study that used mark-recapture of OTC-marked adult *C. carpio* for age validation purposes were based on thin otolith sections (Brown *et al*., 2004). To allow for comparison, OTC-marked otoliths were, therefore, not only examined whole but also sectioned. One asteriscus otolith per specimen was therefore embedded in clear polyester casting resin, sectioned transversely through the nucleus to a thickness of 0·4 mm and mounted on a microscope slide using DPX mounting agent. Whole and sectioned otoliths were viewed under reflected fluorescent (460–490 nm and 510–550 nm) and transmitted white light to determine the position of fluorescent mark and to count the number of opaque zones distal to the mark. To count the total number of growth zones, otoliths were also assessed under transmitted white light using varying magnifications (×10 to ×40).

EDGE AND LENGTH-FREQUENCY ANALYSIS

To corroborate the results from the OTC marking experiment for the wild population, two indirect validation methods were applied: (1) edge analysis and (2) length-frequency analysis (LFA). For both methods, the null hypotheses that growth zone deposition is annual (H_0^1) and that growth zone deposition occurs biannually (H_0^2) were tested. A significance level of $P \leq 0{\cdot}05$ was used for all tests.

A total of 607 otolith pairs were used for the edge analysis. Otoliths with fewer than two growth zones were excluded from the analysis (Vilizzi & Walker, 1999). The optical appearance of the edge of each whole otolith was assessed and categorized as either optically opaque (1) or translucent (0). Edge analysis is based on the assumption that the relative frequency of opaque edges was periodic when plotted against time (Campana, 2001). Otolith samples collected between November 2006 and May 2008 were assigned to 24 monthly periods, M_i, of any given year (with beginning of January being assigned 0 and end of December 23). The binary results were then expressed as the proportion of otoliths with an opaque zone present during M_i and modelled using a periodic logistic regression (Flury & Levri, 1999) of the form $\text{logit}(\hat{O}_i) = \beta_0 + \beta_1 \sin(2\pi M_i P^{-1}) + \beta_2 \cos(2\pi M_i P^{-1})$, where 'logit' is the link function for the binomial regression, $\hat{O}_i$ is the expected proportion of otoliths with an opaque zone present at the margin for each M_i, P is the assumed periodicity of growth zone deposition (*i.e.* here 24 and 12 for an annual and biannual cycle, respectively) and β_0, β_1, β_2 the regression coefficients (Beamish *et al*., 2005).

Regression parameters were estimated by minimizing the binomial negative log-likelihood function of the form $-\ln L = -\sum_i [m_i \ln(\hat{O}_i) + (n_i - m_i)\ln(1 - \hat{O}_i)]$, where n_i is the number of otoliths examined in sample i and m_i represents the number of otoliths with an opaque zone present on the margin. To test H_0^1 and H_0^2, a likelihood ratio test $\chi^2 = -2(\ln L_{red} - \ln L_{full})$ was conducted, where $\ln L_{red}$ is the log-likelihood for the reduced model with the constraint that P is fixed (at either 24 or 12), $\ln L_{full}$ is the log-likelihood for the full model where P is estimated.

Length-frequency analysis was conducted based on a length-based age-structured model suggested by MacDonald & Pitcher (1979). Three large length-frequency (LF) samples were used that were collected during angling competition events in November 2006 ($n = 692$), January 2007 ($n = 297$) and April 2007 ($n = 979$). Sub-samples of otoliths were available for all three LF samples. For each sample, results from LFA were compared to the assumed mean length-at-age (according to H_0^1 and H_0^2) from otolith data.

For each of the samples, length data were regrouped into 10 mm L_F classes i. The resulting LF histograms showed several discernable modes and were therefore considered as suitable. The number of identifiable modes A_s for each LF sample s was graphically determined. For each sample s, it was assumed that the relative proportion of fish observed in length class i, $q_{i,s}$, was equal to the sum of all identifiable modes at length i. If each mode a in sample s can be described by a normal distribution function $\theta(\hat{\mu}_{a,s}, \sigma^2_{a,s})$, where $\hat{\mu}_{a,s}$ and $\sigma^2_{a,s}$ are the mean and the variance, respectively, then the predicted proportion for length class i in sample s can be estimated as $\hat{q}_{i,s} = \sum_{a=1}^{A_s} \hat{p}_{a,s}\theta(\hat{\mu}_{a,s}, \sigma^2_{a,s})$, where A_s is the maximum number of modes in sample s and $\hat{p}_{a,s}$ is the predicted relative abundance of fish in mode a from sample s, such that $\sum_{a=1}^{A} \hat{p}_{a,s} = 1$. The maximum likelihood for the parameters of interest, $\hat{p}_{a,s}$, $\hat{\mu}_{a,s}$ and $\hat{\sigma}_{a,s}$, was obtained for each LF sample by minimizing the robust log-likelihood function (Fournier *et al*., 1990; Simon & Booth, 2007) of the form $\ln L = \sum_{i=1}^{L_s} [\ln(\sqrt{\xi_{i,s}}) + 2\xi_{i,s}^{-1}(q_{i,s} - \hat{q}_{i,s})^2]$, where $\xi_{i,s} = \hat{q}_{i,s}(1 - \hat{q}_{i,s})n_s^{-1}$ is the relative binomial variance of $\hat{q}_{i,s}$, n_s is the number of observations in sample s and L_s is the maximum number of length classes in sample s.

To test H_0^1, mean length-at-age from otolith sub-samples, assuming annual growth zone deposition (*e.g.* 3 years = three opaque zones), was pair-wise regressed against predicted mean length-at-age from LFA. This regression was repeated based on the H_0^2 assumption that growth zone deposition occurs biannually (*e.g.* 3 years = five or six opaque zones, *i.e.* this hypothesis implies that a second opaque zone is deposited at some point during the course of the year and that this year class may therefore include fish with either five or six opaque zones). The assumption of a 1:1 relationship for each of the two regressions was tested using linear regression t-tests previously described. Age-bias plots (Campana *et al*., 1995) were used for each LF sample for further graphical interpretation.

RESULTS

Throughout the study period, >2500 *C. carpio* were measured and 816 otolith pairs were removed. Angling competition surveys were the most effective sampling method and resultant data accounted for >86% of measured fish and 81% of collected otoliths.

ACCURACY AND PRECISION OF OTOLITH INTERPRETATION

Of the 816 otolith pairs analysed, 34 (4·16%) were rejected as unreadable. Estimated APE index for the primary reader was 5·54% and the mean C.V. was calculated as 7·03%. Discontinuities, short and narrow opaque zones, also referred to as 'pseudoannuli' (Vilizzi & Walker, 1999), were clearly distinguishable from the usual thick and continuous 'true' opaque zones and therefore excluded from growth

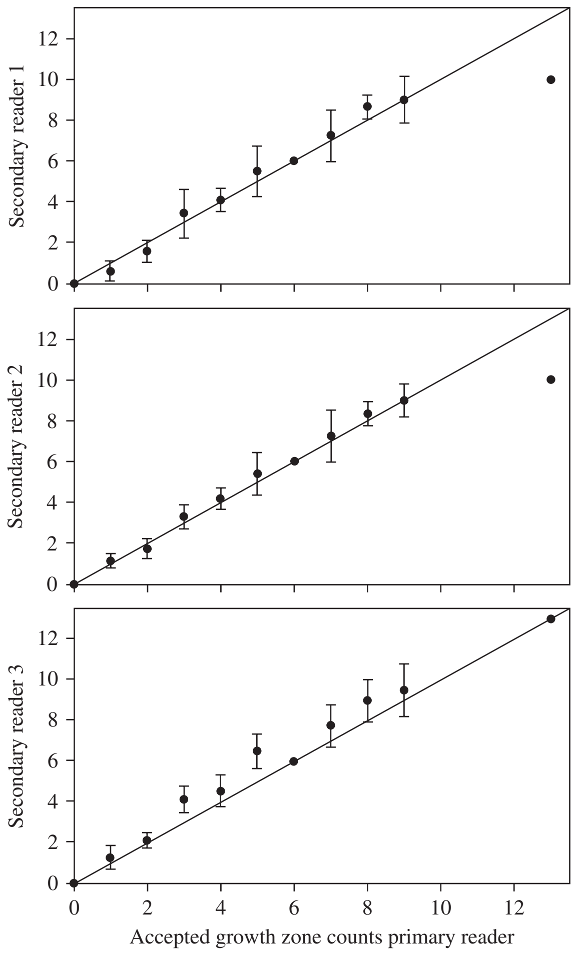

FIG. 3. Age-bias plots showing pair-wise comparison between the growth zone counts accepted by the primary reader and the results from the three secondary readers for whole asteriscus otoliths of *Cyprinus carpio* from Lake Gariep, South Africa. Values are means ± S.D. The solid line in each plot represents a 1:1 relationship ($n = 100$).

zone counts. The maximum number of growth zones counted was 14. Due to a progressive decrease in the width of the translucent zones, interpretations of otoliths with more than eight growth zones became increasingly difficult.

There was no systematic bias between the primary reader and the three secondary readers for the first zero to nine growth zones (Fig. 3), which is the range of growth zones that accounted for 95% of the total sample ($n = 816$). Only the oldest fish in the sample differed between the primary and two of the secondary readers by more than two growth zones. Secondary reader 3 tended to overestimate growth zones compared to the other readers (Fig. 3). The null hypothesis that the slope of the regression = 1 failed to be rejected ($P > 0{\cdot}05$) for all t-tests between the primary reader and the secondary readers and none of the intercept estimates was significantly different from zero ($P > 0{\cdot}05$).

TABLE I. Summary of *Cyprinus carpio* that were injected with oxytetracycline hydrochloride and recaptured after 14 months in large earthen ponds under ambient conditions. The summary includes fork length (L_F)-at-capture (L_{CA}), L_F-at-recapture (L_{RE}), increase in L_F (ΔL), the total number of observed growth increments and those that were deposited prior (F_{PRE}) and posterior (F_{POST}) to the fluorescent mark. Edge appearance was categorized as translucent (T) or opaque (O)

	Length (mm)			Whole otolith				Sectioned otolith			
Tag	L_{CA}	L_{RE}	ΔL	F_{PRE}	F_{POST}	Total	Edge	F_{PRE}	F_{POST}	Total	Edge
Yes	345	530	185	4	2	6	T	4	2	6	T
Yes	345	565	220	5*	2	7	O	5*	2	7	O
Yes	340	536	196	4*	2	6	T	4	2	6	T
Yes	460	575	115	6	2	8	T	6	2	8	O
Yes	295	435	140	N/A	N/A	N/A	N/A	6*	2	8	O
Yes	388	576	188	4	2	6	T	N/F	N/F	6	T
Yes	335	520	185	4	2	6	T	N/F	N/F	U/R	U/R
No	—	586	—	N/A	N/A	N/A	N/A	5	2	7	T
No	—	592	—	6	2	8	T	6	2	8	T
No	—	514	—	4	2	6	T	4	2	6	T
No	—	584	—	4	2	6	T	4	2	6	T
No	—	632	—	8	2	10	T	7	2	9	T
No	—	626	—	6	2	8	T	8	2	10	T
No	—	619	—	4*	1	5	T	N/F	N/F	5	T
No	—	610	—	4	2	6	T	4	2	6	T
No	—	570	—	6	2	8	T	5	2	7	T
No	—	582	—	4	2	6	T	N/F	N/F	U/R	U/R
No	—	520	—	4	2	6	T	4*	2	6	T
No	—	601	—	6	2	8	T	6	2	8	T

N/A, otolith not available; N/F, no fluorescent mark discernable; U/R, unreadable.
*Opaque zone coincided with the fluorescent mark.

FLUOROCHROME MARKING EXPERIMENT

Of the 21 fish that were tagged, injected with OTC and released into the pond in March 2007, 19 were recaptured in May 2008 and only seven (37%) retained the T-bar tag. During the 14 months of the experiment, these specimens grew by an average of 176 mm (95% CL = 141–202 mm) (Table I). Average increase in mass was 1·68 kg (1·37–2·19 kg). The fastest growth was recorded for a specimen that grew 220 mm, from 345 to 565 mm L_F, corresponding to an increase in mass of 2·19 kg.

One of each of the extracted 19 otolith pairs was sectioned and the other was left whole. During the course of the analysis, two of the whole otoliths broke and had to be excluded (N/A). A fluorescent mark was visible in all 17 remaining whole otoliths and in 15 (78%) of the otolith sections (Fig. 4), of which two were classified as unreadable (U/R) due to the absence of clearly discernable opaque zones (Table I). Growth zone counts at the time of recapture ranged from five to 10. If an opaque zone coincided with the fluorescent mark, it was counted as prior to the mark. All but one of the whole otoliths and all of the sectioned otoliths showed two growth zones distal to the OTC mark. The optical appearance of the edge was categorized

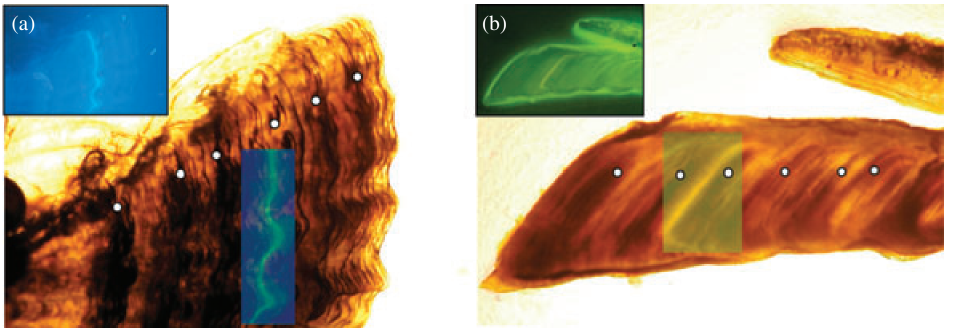

FIG. 4. Photomicrographs using (a) whole and (b) sectioned asteriscus otoliths of *Cyprinus carpio* injected with oxytetracycline 14 months prior to the recapture. The base images show the otoliths under transmitted light with positions of opaque zones (○) indicated. The overlaid portions were viewed with transmitted fluorescent light highlighting the fluorescent mark. Insets show the same images using fluorescent light only.

as translucent for 94 and 82% of the whole and sectioned otoliths, respectively. The results from this experiment suggested that two opaque zones were deposited during the 14 month period in the ponds. The presence of a translucent zone on the edge of the majority of otolith samples at the time of recapture indicated the commencement of a new growth zone.

EDGE AND LENGTH-FREQUENCY ANALYSIS

A biannual periodic regression model best described the temporal frequency distribution of opaque zone deposition on the edge of otoliths over a 1 year period (Table II). Observed and predicted data indicated that the first mode peaked in summer (December to March) and a second mode in winter (June to September; Fig. 5). A likelihood ratio test revealed that there was no significant difference between the full model, where the yearly periodicity was estimated, and a model with a hypothesized deposition of two growth zones per year (H_0^2; $\chi^2 = 0{\cdot}15$, d.f. $= 1$, $P > 0{\cdot}05$).

TABLE II. Parameter estimates from the logistic periodic regression analysis predicting the temporal proportion of opaque zone deposition over a 1 year period for *Cyprinus carpio* in Lake Gariep, South Africa. The periodicity (P) was estimated for the full model and fixed for the unimodal and bimodal model

	Periodic regression models		
Parameter	Annual	Biannual	Full
β_0	−0·61	0·15	0·14
β_1	1·41	1·01	0·83
β_2	1·42	0·96	1·08
P	24·00	12·00	12·20
d.f.	3	3	4
ln$L$	−354·55	−337·57	−337·49

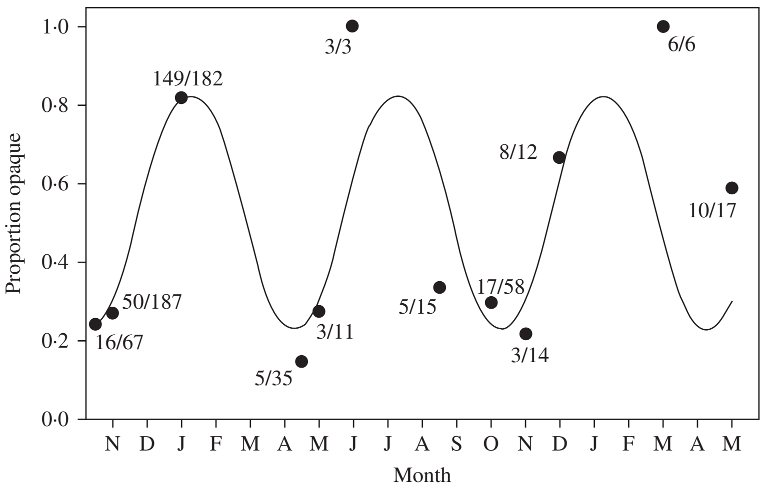

FIG. 5. Proportion of otoliths with an opaque zone on the margin for *Cyprinus carpio* based on data collected between November 2006 and May 2008 from Lake Gariep, South Africa. —, the predicted bimodal annual cycle of opaque zone deposition using a binomial periodic regression. Opaque margins and total number of otoliths examined are denoted as numerator and denominator, respectively.

The null hypothesis that growth zone deposition follows a unimodal annual cycle was, however, rejected (H_0^1; $\chi^2 = 34{\cdot}12$, d.f. $= 1$, $P < 0{\cdot}05$).

Results from LFA are shown in Fig. 6. The November LF sample consisted of a wide range of larger size classes [Fig. 6(a)]. Smaller size classes were absent in this sample and only recruited into angling competition catches during subsequent events in January and April [Fig. 6(b),(c)]. Hence, the first discernable mode observed in the November sample was assigned to age class 2 [Fig. 6(a)]. Based on the number of discernable modes, mean L_F-at-age could be estimated for 5 (November), 3 (January) and 3 (April) sequential age classes, respectively. If growth zone deposition rate was assumed biannual (H_0^2), this finding was identical with the estimated age classes from otolith samples, with the only exception of an estimated 6 year-old specimen from the January sample. In contrast, the annual growth zone deposition hypothesis (H_0^1) resulted in much wider ranges of age estimates (3 to 14 years for November, 1 to 11 years for January and 1 to 6 years for April).

A graphical inspection using age bias plots showed that mean L_F-at-age estimates based on H_0^2 approximated a 1:1 relationship when compared with the results from LFA, whereas estimates based on H_0^1 tended to underestimate the predicted mean L_F-at-age from LFA [Fig. 6(d)–(f)]. The first age class predicted by the LFA for January and April [Fig. 6(b), (c)] exclusively corresponded to L_F groups of *C. carpio* that had otoliths with one or two opaque zones. While only 20% of these fish showed a second opaque zone by January, 50% of fish were recorded with a second opaque zone by April. This means that the results from the LFA provide corroborative evidence that the predicted age 1 year group deposited two opaque zones during the course of 1 year and that fish with one or two opaque zones were part of the same age group. This also implies that not all fish in this age group had deposited the second opaque zone by the time of the April sampling event. The assumptions of

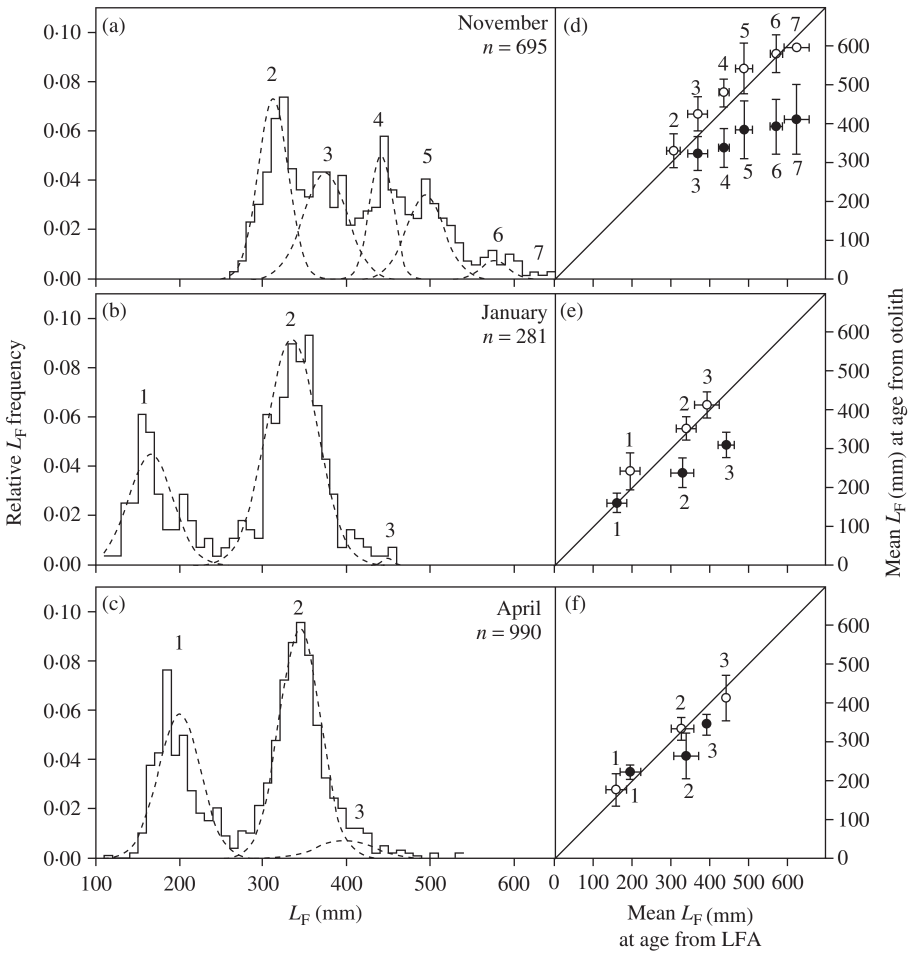

FIG. 6. Length-frequency analysis (LFA) applied to *Cyprinus carpio* angling competition samples from (a) November 2006, (b) January 2007 and (c) April 2007. Solid curves show maximum likelihood fits of mixture distributions with numbers denoting the estimated age groups. (d)–(f) Age-bias plots show corresponding pair-wise comparisons between mean ± S.D. fork length (L_F)-at-age from LFA and otolith data assuming either annual (●) or biannual (○) growth zone deposition rates. - - -, a 1:1 relationship.

a 1:1 relationship (slope = 1, intercept = 0) across all three samples were rejected for H_0^1 (t-tests, $P < 0{\cdot}05$) and were failed to be rejected for H_0^2 (Fig. 7; t-tests, $P > 0{\cdot}05$).

GROWTH ZONES

Growth zone counts based on the number of observed opaque zones were plotted against L_F and grouped according to an assumed biannual growth zone deposition rate that was supported by the concurring results from the three validation approaches (Fig. 8). The number of opaque zones revealed an alternating growth pattern. While larger increases in L_F were observed between even and odd numbers of opaque

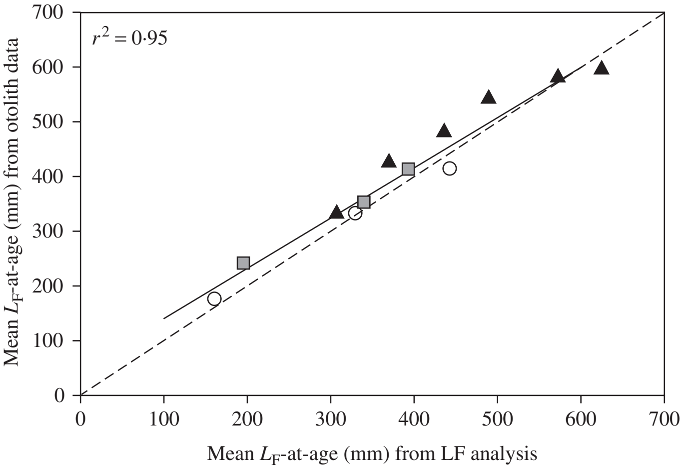

FIG. 7. Pair-wise comparison of mean fork length (L_F)-at-age from the length-frequency analysis (LFA) and mean L_F-at-age from otolith based on the null hypothesis that growth zone deposition occurs biannually (H_0^2). Linear regression: $F_{1,10}$; slope $= 0{\cdot}92$; intercept $= 47{\cdot}8$ mm; $r^2 = 0{\cdot}95$). —, the fit of the linear regression; - - -, a 1:1 relationship. November 2006 (▲), January 2007 (○), April 2007 (■).

zones, growth appeared to be reduced between odd and even numbers (Fig. 8). The most apparent transition in the proportion of odd to even numbers was observed in large otolith sub-samples available for November 2006 and January 2007. When both samples were combined, fish with three and four growth zones formed a dominant group that accounted for 58% of the sample ($n = 450$). While 83% of otoliths sampled in November ($n = 99$) were observed with three opaque zones, this proportion changed to 91% of this group ($n = 162$) having four opaque zones when sampled in January, suggesting that these fish were likely to be part of the same age group.

DISCUSSION

Fish otoliths have been described as metabolically inert environmental recorders, with their microstructure being controlled by a suite of physical, chemical, environmental and physiological factors (Campana, 1999). This may result in regional differences of growth zone deposition rates and can therefore influence ageing precision or the choice of otolith preparation technique. Additional complications arise from the effect of reader subjectivity, which might be reduced and quantitatively determined, but not eliminated (Campana *et al.*, 1995). Therefore, the findings of this study should be interpreted as system- or region-specific.

In a comprehensive study on ageing *C. carpio* in the Murray-Darling Basin, Australia, Vilizzi & Walker (1999) found comparable interpretabilities between whole and sectioned asteriscus otoliths, which they related to the specific morphological characteristics of these otoliths in cyprinids. Moreover, they noted that underestimation of the ages of older *C. carpio* using whole otoliths instead of sections, a phenomenon often demonstrated for sagittal otoliths (Dwyer *et al.*, 2003), was not evident, and that sectioning otoliths even obscured the visibility of opaque zones close to the margin. Similar problems with sectioned otoliths were encountered during exploratory otolith preparation for the present study and the highest confidence in the optical interpretation of asteriscus otoliths was achieved if they were read whole, immersed in methyl salicylate and viewed in transmitted light. In some instances, however, sectioned otoliths may be more suitable or convenient. Brown *et al.* (2004), for example, reported higher within-reader precision for sectioned otoliths (APE = 4·04–4·56%; C.V. = 5·55–6·41%) than found in this study (APE = 5·54% and C.V. = 7·03%), which were similar to those reported by Vilizzi & Walker (1999) for whole otoliths (APE = 5·64%; C.V. = 7·97%). While some of the differences in precision and choice of ageing structures may be a result of the experience and preference of the interpreter (Brown *et al.*, 2004), others are likely to be caused by differences in the optical properties among populations (Jackson & Quist, 2007).

The between-reader comparison experiment was conducted to test for the statistical significance of potential systematic biases that may have been related to false ageing criteria. The requirement for such an approach is that the secondary reader needs to be naive about the species-specific hard structure of interest, but should also be experienced in ageing other fish species. A disadvantage is that this experiment is not suitable for determining between-reader precision, particularly when considering that *C. carpio* was described as difficult to age species that requires extensive training and experience (Vilizzi *et al.*, 1998). The results suggest that all three secondary readers spontaneously applied similar interpretation criteria to those established by the primary reader after several preliminary reading trials and a study of available literature. This indicates that accepted otolith readings in the range between zero and nine growth zones are likely to have a high degree of reproducibility.

Among the three validation approaches applied in this study, chemical marking of wild fish is considered most powerful (Campana, 2001; Dwyer *et al.*, 2003; Brown *et al.*, 2004; Egger *et al.*, 2004). Although it was not possible to conduct this experiment in the lake itself, the results are considered to have strong implications, in

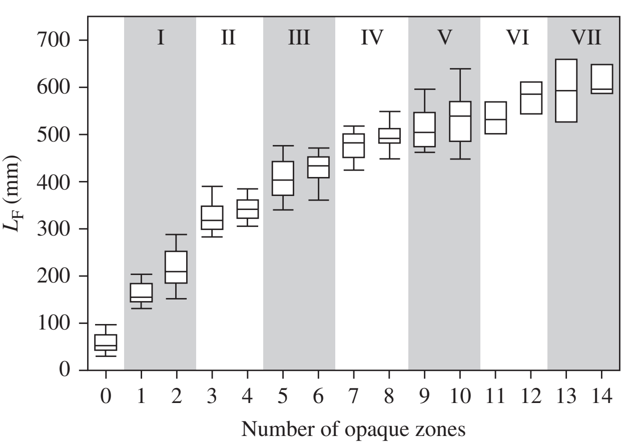

FIG. 8. Box and whisker plots derived from observed lengths at opaque zones. The alternating □ and ■ highlight the grouping in age classes according to an assumed biannual deposition of growth zones with roman numbers denoting the age groups ($n = 782$).

particular since the wild-caught *C. carpio* were subject to the same seasonal changes in ambient temperatures, rainfall and day length regimes during their 14 months at liberty in the large ponds.

Although edge and marginal increment analysis belong to the most frequently used validation methods, they have been criticized for their lack of objectivity concerning the graphical interpretation of the results (Campana, 2001). The logistic periodic regression approach (Flury & Levri, 1999; Beamish *et al.*, 2005; Winker *et al.*, 2010) allows modelling an assumed sinusoidal periodicity. As the parameter estimates are based on the minimization of the negative binomial log-likelihood function, monthly observations of proportions of opaque zones on the margin are weighed according to sample sizes, a factor which is extremely difficult to quantify and incorporate into a graphical assessment. Furthermore, the likelihood approach provides the opportunity to objectively test the null hypotheses of whether the deposition rate follows a unimodal or bimodal yearly cycle. As the choice of distributions and related log-likelihood functions (*e.g.* binomial, normal, lognormal, gamma and Poisson) is in principle flexible, this approach also has the potential to be applied to measurement results from marginal increment analysis.

A prerequisite for using length-frequency analysis to corroborate results from other validation methods is that length modes should be discernable, which therefore limits the range of applications to relatively fast-growing fishes or younger age groups (Campana, 2001). Given that the data are suitable, inferences from subsequent comparisons, for example, of monthly length modes in juvenile fishes or with estimates obtained from ageing structures may add valuable supporting or contradicting information (Jensen, 1970; Cochrane, 1985; Campana, 2001; Dwyer *et al.*, 2003; Brown *et al.*, 2004). The maximum likelihood estimation procedure (Fournier *et al.*, 1990; Simon & Booth, 2007) is able to provide a unique and statistically robust solution for a single length-frequency distribution. Any comparison with otolith-derived length-at-age data should, however, be based on length-frequency data and corresponding sub-samples of otoliths that were collected at random from the same sampling event, so that factors such as catchability, size-selectivity, changes in spatial distributions and modal growth progression are more likely to be minimized.

All three applied validation approaches suggest that the deposition of growth zone increments occurs biannually. Supporting evidence for the possibility of a biannual growth zone deposition rate in the Lake Gariep population comes from a study by Cochrane (1985), which was conducted in Hartebeespoort Dam, a South African hypertrophic impoundment situated *c*. 580 km north-east of Lake Gariep. Cochrane (1985) inferred a formation of two growth zones per year in the vertebrae of *C. carpio* by comparing mean length-at-age estimates derived from available length-frequency samples using the Petersen (1891) method to calculate time of growth zone formation.

The predicted increase in the proportion of opaque zones in whole otoliths between October and January (Fig. 5) was also noted by Vilizzi & Walker (1999) and is in accordance with the time of growth check formation in vertebrae that was reported for the *C. carpio* population from Hartebeespoort Dam (Cochrane, 1985). This period of late spring and early summer usually coincides with peak spawning events of *C. carpio* (Cochrane, 1985; Fernàndez-Delgado, 1990; Sivakumaran *et al.*, 2003; Smith & Walker, 2004; Brown *et al.*, 2005). This was noticeable in this study as a large proportion of reproductively active *C. carpio* were recorded during November

2006. Fernàndez-Delgado (1990) also noted a sharp decrease in the somatic condition of *C. carpio* during this period, which they ascribed to high energy requirements for spawning.

The second opaque zone formation was predicted to occur during mid-winter (Fig. 5) when surface temperatures were at a minimum (8·7–11·3° C). This also agrees with the timing of the annual opaque formation found in whole asteriscus otoliths of two large endemic cyprinids, *Labeo capensis* (Smith) and *Labeobarbus aeneus* (Burchell), which co-occur with *C. carpio* in Lake Gariep (Winker *et al*., 2010). In Southern Africa, Hecht (1980) and Weyl & Booth (1999) observed biannual formation patterns similar to this study in sagittae otoliths of the tilapia species *Oreochromis mossambicus* (Peters) and on scales of the cyprinid *Labeo cylindricus* Peters with one formation evident by the end of the peak spawning period and a second in winter when temperatures were low.

While the underlying processes of opaque zone depositions remain largely unclear and can be influenced by environmental variability, protracted spawning or a delay in the optical recognition of opaque material (Francis *et al*., 1992; Vilizzi & Walker, 1999; Smith & Deguara, 2003; Egger *et al*., 2004; Bwanika *et al*., 2007), it appears to be a reasonable explanation that the biannual growth zone deposition in otoliths of adult *C. carpio* from Lake Gariep is related to high investment in reproduction during early summer and to low water temperatures in winter.

Most uncertainty remains over the formation of the first two opaque zones because their deposition rate was not validated by fluorescent marking. Vilizzi & Walker (1999) found that otoliths with one and two increments showed almost exclusively translucent margins throughout the year, which they explained by a rapid development of the otolith structure in young fish in combination with relatively thin first opaque zones. This is similar to observations from the present study. Otoliths with one growth zone, for example, were excluded from edge analysis because they obscured the periodic pattern observed for older fish due to their high proportions of translucent margins. A weakly defined periodic pattern (not shown here) indicated that a first opaque zone was primarily formed between May and June, presumably signifying the end of the first growing season, whereas hardly any otoliths were noted with a single opaque zone on the margin of otoliths sampled between October and April. Otoliths with two opaque zones were generally scarce in all samples. In contrast to otoliths with a single increment, however, this two opaque zone group was predominantly found with opaque zone margins during the summer months, which agreed with the predicted periodicity of opaque zone deposition for older fish (Fig. 5). Under the assumptions that growth slows down as fishes become older and that the initial somatic growth of immature fishes is fastest because no energy needs to be allocated to reproduction (Roff, 1984; Shuter *et al*., 2005), the presented results favour the hypothesis that growth zone deposition during the first year of life also occurs biannually. This is supported by the following corroborative evidence: (1) specimens with one and two growth zones were both part of the first LF modes observed in January and April 2007, which was clearly distinct by a wide gap from subsequent LF modes (Fig. 6) and (2) the relative increase in L_F between the first and the second increment was smaller than the increase between the second and third (Fig. 8).

Although the data provide some support for a biannual growth zone deposition rate, the argument that one of these growth zones is formed as a result of energy reallocation towards reproduction is obviously not valid for immature fish. Thus, the possibility of an annual deposition rate cannot be excluded. The formation of a growth increment in immature specimens during the spawning season, however, could be linked to the reported size-dependent seasonal habitat shift of *C. carpio* (García-Berthou, 2001; Penne & Pierce, 2008). In a recent telemetry study on the movement of *C. carpio* in Lake Crescent, Iowa, U.S.A., Penne & Pierce (2008) showed that larger *C. carpio* (>400 mm L_F) mainly aggregate in deeper waters (>2 m) during autumn and winter and only move into the shallow littoral zone (<2 m) before and during spawning, whereas smaller specimens (220–300 mm L_F) predominantly resided in the shallow littoral zone (0·5–1·5 m) throughout the year. This behaviour of larger specimens may therefore result in an increased competition for food in this habitat and cause a nutritional bottleneck for smaller specimens, in particular when foraging behaviour of mature specimens is potentially intensified between spawning events. Further research, however, is required to investigate the processes associated with the timing of increment formation and in particular that of the first two opaque zones.

In summary, this study provides sufficient evidence that growth zone formation is biannual in asteriscus otoliths of age 2+ years *C. carpio* from Lake Gariep and represents the first validation of growth zones in otoliths conducted on an African *C. carpio* population. Given that increments in the same ageing structure were validated as annual for *C. carpio* populations of the Murray-Darling Basin, Australia (Vilizzi & Walker, 1999; Brown *et al*., 2004), the findings demonstrate that growth zone deposition rates can vary among populations of the same fish species and exemplifies the importance of age validation as a prerequisite for understanding the life history of *C. carpio* in different geographical localities.

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