Testing the assumption of annual shell ring deposition in freshwater mussels

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Abstract: We tested the assumption of annual shell ring deposition by freshwater mussels in three rivers using 17 species. In 2000, we notched shell margins, returned animals to the water, and retrieved them in 2001. In 2003, we measured shells, affixed numbered tags, returned animals, and retrieved them in 2004 and 2005. We validated deposition of a single internal annulus per year in all species and in 94% of specimens. Most unvalidated shells were old individuals with tightly crowded rings. Handling produced a conspicuous disturbance ring in all specimens and often resulted in shell damage. Observed growth was similar to but slightly lower than growth predicted by von Bertalanffy length-at-age models developed independently from shell annuli; further, handling specimens in 2 consecutive years reduced growth more than handling only once. These results show that mussels are extremely sensitive to handling. Brief handling does not likely increase short-term mortality, but repeated handling could decrease long-term fitness. Handling effects should be considered in sampling programs or when interpreting results of mark–recapture studies designed to estimate mussel growth. Production of annual shell rings is a pervasive phenomenon across species, space, and time, and validated shell rings can provide accurate estimates of age and growth.

Résumé : Nous testons la présupposition qui veut que la déposition de l'anneau dans la coquille des moules d'eau douce soit annuelle chez 17 espèces dans trois rivières. En 2000, nous avons entaillé la bordure des coquilles, retourné les moules à l'eau et récupéré les animaux en 2001. En 2003, nous avons mesuré les coquilles, fixé des étiquettes numérotées et retourné les animaux à l'eau pour les récupérer en 2004 et 2005. Nous avons confirmé la déposition d'un seul annulus interne par an chez toutes les espèces et 94 % des spécimens. La plupart des coquilles pour lesquelles nous n'avons pas réussi à faire de confirmation appartenaient à des individus âgés avec des anneaux serrés les uns contre les autres. La manipulation produit un anneau de perturbation bien visible chez tous les spécimens et souvent endommage la coquille. La croissance observée est semblable, bien qu'un peu inférieure, à celle prédite par les modèles de longueur en fonction de l'âge de von Bertalanffy et calculée indépendamment des annulus de la coquille; de plus, une manipulation durant 2 années consécutives réduit la croissance plus qu'une seule manipulation. Ces résultats démontrent que les moules sont très sensibles à la manipulation. Une courte manipulation n'augmente probablement pas la mortalité à court terme, mais des manipulations répétées pourraient réduire la fitness à long terme. Les effets de la manipulation doivent donc être pris en considération dans les programmes d'échantillonnage et lors d'interprétations d'études de marquage–recapture destinées à estimer la croissance des moules. La production d'anneaux annuels sur la coquille est un phénomène répandu chez toutes les espèces dans l'espace et dans le temps et des anneaux de la coquille qui ont été confirmés peuvent fournir des estimations précises de l'âge et de la croissance.

[Traduit par la Rédaction]

Introduction

In temperate regions, formation of annual rings in hard, permanent structures in response to cessation or decrease in growth during winter is a pervasive phenomenon in woody plants and poikilothermic animals. Information from these rings forms the basis of our understanding of age, growth, and longevity for many organisms. In marine and estuarine bivalves, the formation of annual winter rings and other types of shell rings, including disturbance marks, spawning marks, and daily growth increments, has been demonstrated and validated for a large number of species, and analysis of rings has been refined to a high degree. Shell ring data from marine bivalves are used widely to construct generalized growth models (Murawski et al. 1982; Jones et al. 1990), examine temporal and geographic variation in growth (Richardson et al. 1990; Rice and Pechenik 1992), determine timing of spawning events (Jones 1980), and make inferences about past events and environmental conditions (Rhoads and Pannella 1970; Jones 1981; Quitmyer and Jones 1997).

The presence of regularly spaced rings in freshwater mussel shells has been noted by biologists for at least 150 years (see Chamberlain 1931), but debate over whether these rings are deposited annually persists to the present day (e.g., Isley 1914; Kesler and Downing 1997). Conspicuous rings or bands appear on the external shell surface, within shell

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cross-sections, and within the shell hinge ligament. External rings are clearly visible on young, fast-growing shells of many species, and the number of external rings is highly correlated with the number of internal rings in these specimens (Neves and Moyer 1988; Veinott and Cornett 1996; Rogers et al. 2001). In older specimens, external growth rings are too crowded and obscured to be interpreted and counted reliably and are difficult to distinguish from other, non-annual rings thought to be deposited in response to disturbance (Neves and Moyer 1988). For these reasons, microscopic examination of internal shell rings in thin section is assumed to provide more precise, less ambiguous estimates of age (Neves and Moyer 1988; Veinott and Cornett 1996). However, the assumption of annual formation has rarely been demonstrated conclusively for either external or internal shell rings.

The handful of studies that have attempted to test the assumption of annual shell ring formation vary widely in their conclusions and their conclusiveness. Mark–recapture studies focusing on external shell rings showed that some marked specimens deposited a single winter ring between growing seasons, but other specimens were not interpretable or produced ring patterns not supportive of the hypothesis of annual formation (Negus 1966; Haukioja and Hakala 1978; Downing et al. 1992). Only a single study has shown consistent, annual formation of external rings (Ghent et al. 1978). The equivocal results of most studies underscore the low precision and interpretability of external rings and support internal rings as more useful indicators of age. Coker et al. (1921) present convincing evidence for annual formation of both internal and external rings, but these observations were anecdotal and from a small number of specimens. Annual formation of internal shell rings and bands in the hinge ligament is well accepted for *Margaritifera margaritifera* but is based on unpublished work (al-Mousawi 1991 in Hastie et al. 2000). Similarly, Howard and Cuffey (2006) reported validation of annual ring formation in *Margaritifera falcata* but did not present specific results of this component of their study. In *Elliptio complanata*, peaks in the concentration of shell $\delta^{18}O$, which are deposited at low temperature, coincided with the location of presumed internal winter annuli, providing strong support for annual ring formation, even though the study involved only five individuals, and one out of eight rings identified initially as annuli was evidently not deposited in winter (Veinott and Cornett 1996). Neves and Moyer (1988) conducted a validation study for several species but confirmed annual internal ring formation in only 12% of specimens; however, failure to validate most specimens was due to an inability to locate the position of their reference mark within the tightly crowded shell rings of older specimens, and no interpretable specimens showed evidence contrary to the assumption of annual formation. Only one study has seriously challenged the assumption of annual formation of internal shell rings, based on poor agreement between growth rates predicted by analysis of internal rings and growth rates observed in a mark–recapture study (Kesler and Downing 1997). Together, this small and contradictory body of research provides conclusive support for annual formation of shell rings in few species and allows no consensus about the generality of this phenomenon.

Even in well-studied organisms, variations in growth among species, habitats, and age classes warrant critical evaluation of any aging method based on interpretation of rings in hard structures. Beamish and McFarlane (1983) criticized the lack of validation in many age studies of fishes and illustrated some of the serious errors that can result from misinterpretation of unvalidated indicators of age. The pitfall of interpreting age estimates based on unvalidated methods is chronic for freshwater mussels. Many age studies of freshwater mussels based on shell rings offered no support for or evaluation of the assumption of annual ring formation (e.g., Paterson 1985; Hinch et al. 1986; Woody and Holland-Bartels 1993) or cited unpublished data as support (McCuaig and Green 1983; Haag and Staton 2003). Most commonly, age studies have routinely supported the assumption by referencing a common suite of papers (e.g., Isley 1914; Haukioja and Hakala 1978; Neves and Moyer 1988), even though these supporting papers reported equivocal results and usually involved species different from those being studied (e.g., Brown et al. 1938; Bruenderman and Neves 1993; Jones et al. 2004). By not addressing validation or by uncritical citation of previous work, these studies have tacitly elevated an untested assumption to the level of a paradigm (see Horn 2001), leaving the bulk of existing age–growth information for freshwater mussels of questionable validity.

Because many species of North American freshwater mussels are critically endangered and others are important commercially, validated age and growth information as potentially inferred from shell rings will be invaluable to resource managers. In this study, we evaluate deposition of shell rings across multiple years in three rivers in the southeastern United States, using 17 species of freshwater mussels (*Amblema plicata*, *Elliptio arca*, *Fusconaia cerina*, *Fusconaia flava*, *Lampsilis cardium*, *Lampsilis ornata*, *Lampsilis teres*, *Leptodea fragilis*, *Obliquaria reflexa*, *Plectomerus dombeyanus*, *Potamilus purpuratus*, *Pyganodon grandis*, *Quadrula asperata*, *Quadrula pustulosa*, *Quadrula quadrula*, *Quadrula rumphiana*, *Tritogonia verrucosa*). We test the hypothesis that shell rings are deposited annually, describe other types of rings deposited in shells, and assess how shell ring deposition differs among individuals, species, space, and time. We also examine the effect of handling on growth of mussels in mark–recapture experiments designed to test the assumption of annual ring deposition and evaluate the usefulness of this approach as a validation method.

Materials and methods

Study sites

We studied shell rings at one site in the Little Tallahatchie River, Panola County, Mississippi (34°23′56″N, 89°47′33″W), one site in the St. Francis River, Cross County, Arkansas (35°16′12″N, 90°34′58″W), and at one site in the Sipsey River, Pickens–Greene County, Alabama (33°07′16″N, 87°55′08″W). All three sites support diverse and abundant mussel communities, but physical habitat conditions differ greatly among the streams. The Little Tallahatchie River is regulated and impounded; our site was located in the dam tailwater below a major storage reservoir (Sardis Reservoir) and is impounded by a low-head dam about 2–3 km downstream of the site (Haag and Warren 2007). Substrate at the Little Tallahatchie River site was composed primarily of sand, and water depth was approximately 3.0 m. The St. Francis

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River is largely unregulated in the vicinity of our study site, but much of the watershed is affected by channelization and water diversion projects; land use in the watershed is dominated by large-scale, intensive agriculture, and the river receives heavy runoff from these activities (Ahlstedt and Jenkinson 1991). Substrate at the St. Francis River site was mostly silt with some sand, and water depth was 1.0–1.5 m. The Sipsey River is unregulated and unmodified, and the watershed is mostly forested; consequently, water quality is high and the river supports one of the most intact aquatic communities in the region (Haag 2002; McCullagh et al. 2002). Substrate at the Sipsey River site was composed of stable gravel and sand, and water depth was about 1.2 m.

Collection and marking of shells

Our general study approach was to collect mussels, mark them, and retrieve them at least 1 year later to examine shell growth deposited during the intervening time period. At all sites, we collected mussels by snorkeling and using SCUBA and attempted to collect representatives of most species present at the site, including as wide a size range as possible for each species. In each year of the study, we collected animals in about the middle of the growing season so that any shell rings or other features potentially resulting from handling or marking would be spatially distinct from any rings potentially produced by a cessation of growth in winter. For all collections, we attempted to minimize handling effects by keeping mussels submerged in mesh bags until they could be returned to the substrate.

In 2000, we batched marked mussels in the Little Tallahatchie River (11 July, n = 87, five species) and Sipsey River (15 June, n = 327, seven species) by filing a 1–2 mm deep triangular notch in the ventral margin of the shell to produce a known reference mark both on the shell exterior surface and within the interior of the shell (Jones et al. 1978; Richardson 1989; Howard and Cuffey 2006). We also filed a shallow groove in the umbo to allow quick recognition of notched animals. To facilitate later relocation of notched animals, we returned mussels to the stream in open-topped plastic tubs (320 mm × 265 mm × 140 mm) filled with substrate and buried flush with the surface of the stream bottom. Additionally, we drilled holes in the sides of the tubs to allow interstitial flow. Total handling time for specimens, including collection, marking, and return to the stream, did not exceed 3 h. Mussels were stocked in tubs at densities similar to ambient mussel density in the stream at each study site. On 25 October 2000, we collected one notched specimen each of *Amblema plicata* and *Quadrula pustulosa* from tubs in the Little Tallahatchie River to examine shell growth before potential deposition of winter annuli. We retrieved all other mussels from tubs 1 year later (2001) and returned specimens to the laboratory for shell thin-sectioning. In the Little Tallahatchie River (18 July), we recovered a total of 67 live, notched specimens (77%) and thin-sectioned 64 specimens; in the Sipsey River (12 June), we recovered a total of 188 live, notched specimens (58%) and thin-sectioned 93 specimens. We thin-sectioned representatives of all notched species.

In 2003, we marked mussels individually in the Little Tallahatchie (20 August, n = 297, seven species) and St. Francis rivers (22 August, n = 263, nine species) by affixing numbered shellfish tags (Floy Company, Seattle, Washington) to shells using cyanoacrylate glue. We recorded the length (greatest anterior–posterior dimension, nearest 0.1 mm) of each specimen and returned all animals to the stream, placing them in a natural filtering position in the substrate. Total handling time for specimens, including collection, marking, and return to the stream, did not exceed 3 h. To reduce the potential for influencing natural growth rates, we did not notch specimens or confine them in plastic tubs as in the 2000–2001 experiments. Rather, we facilitated relocation by placing animals within a prescribed area delimited by rebar stakes driven into the stream bottom. In 2004 in the Little Tallahatchie River (5 August), we recovered a total of 234 live, tagged specimens (79%), measured and released 142 of these, returned 92 to the laboratory, and thin-sectioned 89 specimens. In 2004, we also tagged and released an additional 99 previously unmarked animals (nine species) encountered while searching for previously tagged specimens in the Little Tallahatchie River. In 2005 (3 August), we recovered and thin-sectioned a total of 68 specimens, including 14 that were tagged in 2003 but not recovered in 2004, 34 recaptures that were tagged in 2003 then measured and released in 2004, and 20 that were tagged initially in 2004. In 2004 (2 August) in the St. Francis River, we recovered and thin-sectioned a total of 43 live, tagged specimens (16%), but did not mark any additional mussels in 2004. We revisited the site in 2005 (4 August) and found six specimens tagged in 2003 but not recovered in 2004. From both rivers, we thin-sectioned representatives of all species tagged initially, with the exception of *Potamilus ohiensis* (specimens tagged in both rivers) and *Quadrula nodulata* (St. Francis River only), for which no live individuals were recovered in 2004 or 2005.

Preparation of shell thin sections

We prepared radial thin sections (~300 μm) from one valve of each specimen (Fig. 1) using a low-speed saw with a diamond-impregnated blade (Buehler Ltd., Lake Bluff, Illinois), based on standard methods for bivalves (Clark 1980; Neves and Moyer 1988; Veinott and Cornett 1996). We cut the valve into two halves along a plane originating at the peak of the umbo thence at a slight diagonal to the ventral margin at a point slightly posterior of the midpoint of the shell (Fig. 1); this plane intersected most growth lines at a right angle (Neves and Moyer 1988). For shells from the 2000 experiment, we made the cut adjacent to the notch, but preserved the notch intact for later reference. We selected one of the resulting shell halves and wet-sanded the cut surface on a series of progressively finer sandpapers (400, 600, and 1500 grit). We then affixed the cut surface to a standard frosted glass microscope slide (25 mm × 75 mm) or a larger unfrosted slide (75 mm × 50 mm), according to the size of the shell, using readily available epoxy cement. We affixed slides to a specimen-mounting chuck by heating the chuck on a hot plate, rubbing a bar of paraffin on the mounting surface of the chuck, then pressing the slide into the molten paraffin. After the paraffin cooled, we mounted the chuck onto the saw cutting arm and cut away all of the shell except for the resulting thin section cemented to the slide. With the slide and thin section still attached to the mounting chuck, we wet-sanded thin sections as described above for the initial cut.

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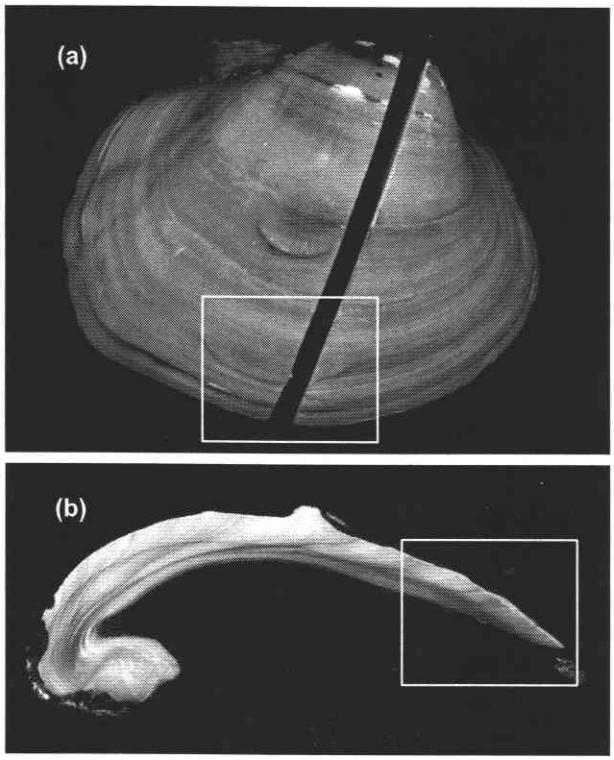
Fig. 1. (*a*) Shell of *Quadrula pustulosa* halved for thin-sectioning and (*b*) resulting thin section. Outlined area shows location of thin sections illustrated throughout this paper.



Interpretation of thin sections

In the 2000–2001 experiments in the Little Tallahatchie and Sipsey rivers, our goal was to use the filed notch in the shell margin as a reference point from which to examine shell rings and other features produced in the year subsequent to notching. On each thin section, we established the location of the shell margin at the time of notching by juxtaposing the cut surface of the shell half containing the notch with the corresponding cut surface of the thin section. With the two shell pieces so aligned, we made a pencil mark on the thin-section slide corresponding to the location of the notch on the shell half. Two experienced observers then read each shell thin section independently and recorded their interpretation of shell features produced during and after notching. We considered the hypothesis of annual ring deposition validated if both readers observed a single ring deposited beyond the notch.

Examination of thin sections from 2003–2005 revealed that handling (even without notching) caused deposition of a disturbance ring in most specimens both internally and on the exterior shell surface, corresponding to the location of the shell margin at the time of handling (see Results). Therefore, we used the disturbance ring as a reference point with which to evaluate the hypothesis of annual ring deposition in 2003–2005 as described for 2000–2001. For specimens tagged in 2003 and recovered in 2004 and for specimens tagged in 2004 and recovered in 2005, we considered the hypothesis of annual ring deposition validated if both readers observed a single ring deposited beyond the disturbance ring caused by handling. For specimens tagged in 2003 and recovered in 2005 (including specimens recovered and released in 2004), we considered the hypothesis of annual ring deposition validated if both readers observed two annuli (representing annuli deposited in the winters of 2003–2004 and 2004–2005) deposited beyond the disturbance ring formed as a result of initial handling in 2003. We expected to see an additional disturbance ring in specimens collected initially in 2003, recovered, measured, and released in 2004, and collected in 2005. All shells collected in 2005 were read blindly without knowledge of their prior collection history or of the location of disturbance rings caused by prior handling. After reading a thin section, we confirmed our identification of the disturbance rings by juxtaposing the thin section with the cut shell as described previously. Because these shells had no notch as a reference point, we located the disturbance rings on the shell surface using calipers to identify the position of the shell margin at the time of handling, based on the shell length recorded for the specimen in 2003 or 2004.

Effects of handling on growth

In the 2003–2005 experiments in the Little Tallahatchie and St. Francis rivers, we compared growth of tagged animals in the wild with growth predictions derived from von Bertalanffy length-at-age models. Length-at-age models were developed using putative internal shell annuli from an independent set of specimens from both study sites (W. Haag, unpublished data). Because we assumed that the brief handling involved in measuring and tagging shells without notching would not result in serious disruptions of growth, we initially expected that comparison of observed and predicted growth would provide an independent test of the annual production of shell rings (see Kesler and Downing 1997). We recovered sufficient tagged specimens for this type of comparison for only six species with existing length-at-age models: *Amblema plicata*, *Obliquaria reflexa*, and *Quadrula pustulosa* (Little Tallahatchie River); and *Lampsilis teres*, *Leptodea fragilis*, and *Potamilus purpuratus* (St. Francis River).

We determined measurement error for *Quadrula pustulosa* by taking 10 replicate length measurements (nearest 0.1 mm) for each of 25 specimens (size range = 23.9–63.0 mm) and computing the variance of the 10 measurements for each specimen. Because there was no relationship between shell length and variance (F = 1.289, 1 df, P < 0.268), we used nested analysis of variance to estimate the overall variance component resulting from within-specimen measurement error among all 25 specimens (s^2 = 0.016, 0.014% of total variance). Using this overall variance estimate, we determined that our 95% confidence limit around an estimated difference between two length measurements was ±0.1 mm (Sokal and Rohlf 1995). We used estimates of measurement error primarily to evaluate potential decrease in size of large specimens of *Quadrula pustulosa*. We did not estimate measurement precision for other species because of small sample sizes and because few other individuals decreased in size.

We evaluated differences in observed and predicted growth in two ways. First, for all six species we computed the predicted length of each specimen in 2004 based on that specimen's initial length (in 2003) using von Bertalanffy growth

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models and then calculated the difference between predicted and observed length for each specimen. We analyzed separately growth of male and female *Potamilus purpuratus* because of strong sexual dimorphism in shell shape. For *Amblema plicata*, *Obliquaria reflexa*, and *Quadrula pustulosa*, we then used a Wilcoxon signed rank test for paired observations to test the hypothesis that observed and predicted size in 2004 did not differ. We present data for *Lampsilis teres*, *Leptodea fragilis*, and *Potamilus purpuratus* but did not conduct statistical tests for these species because of the small numbers and size ranges of recovered individuals. Second, we constructed regression equations describing length in 2004 against length in 2003 (Ford–Walford plots) for both observed and predicted growth. For the predicted growth regressions, we computed the predicted length of each specimen in 2004 based on its length in 2003 using the von Bertalanffy growth models. Because the regression for predicted growth had no variance, we evaluated the similarity between the two growth equations by plotting the 95% prediction interval around the regression line for observed growth and visually assessing the degree to which this interval contained the predicted regression line. We did not construct Ford–Walford plots for *Lampsilis teres*, *Leptodea fragilis*, or *Potamilus purpuratus* because of small sample sizes.

We evaluated the effects of repeated handling of *Quadrula pustulosa* by comparing 2005 length of individuals that were handled twice with 2005 length of individuals that were handled only once. Individuals handled twice were tagged initially in 2003, recaptured, measured, and released in 2004, and recaptured for a second time in 2005. Individuals handled once were tagged initially in 2003, not found in 2004, and recaptured for the first time in 2005. We constructed Ford–Walford plots separately for both handling treatments by plotting final 2005 length against initial 2003 length. Because the slopes of these two relationships did not differ ($F = 0.11$, 1 df, $P < 0.736$), we used analysis of covariance with initial length as the covariate to test for differences in final length between individuals that were handled once and those that were handled twice.

Results

Validation of annuli in 2000–2001

Notching the shell margin resulted in production of a conspicuous disturbance ring visible both internally and on the exterior shell surface in mussels from both the Little Tallahatchie and Sipsey rivers. External disturbance rings appeared as a cleft or shallow groove or as a thin dark line, all of which were readily visible on the shell surface (Fig. 2). Internal disturbance rings intersected the shell surface at the exact location of the external ring. In some cases, internal disturbance rings did not continue throughout the interior of the shell but were visible for only a short distance from the shell surface or were discontinuous within the shell (Fig. 3). In other shells, internal disturbance rings were continuous from the shell surface to the umbonal region (Fig. 4). Internal disturbance rings were usually sharp-edged and very dark, appearing as thin cracks in the shell (Figs. 3 and 4).

In many specimens, external shell abnormalities resulted when shell growth after disturbance resumed at a position offset from the plane of previous growth, resulting in misalignment of the exterior shell surface and prismatic layer (Figs. 4 and 5). Further, periostracum and prismatic shell material deposited immediately prior to disturbance often broke off after being exposed by postdisturbance misalignment of new growth, resulting in shell margin damage and loss (Fig. 6). In some older individuals with low growth rates, shell margin breakage resulted in a reduction of overall length because too little new shell material was deposited after disturbance to extend beyond the location of the original shell margin. Shell margin damage in older specimens was difficult to see except under magnification (Fig. 6). Although younger specimens often sustained similar damage, higher growth rates always resulted in increased shell size. In all specimens, shell loss was limited to periostracum and prismatic shell material, and no loss of nacreous material was observed.

In 2001, the hypothesis of annual ring production was validated in both rivers, for all species, and in 92% of notched specimens that we examined ($n = 157$, Table 1). In all validated specimens, a disturbance ring was produced at the time of initial collection and notching, shell growth resumed in 2000 followed by deposition of a single internal winter annulus, and additional shell growth occurred in 2001 prior to final collection (Figs. 3 and 4*a*). Specimens of *Amblema plicata* and *Quadrula pustulosa* collected from the Little Tallahatchie River in October 2000, approximately 3 months after notching, had each deposited a disturbance ring followed by a resumption of shell growth, but no internal or external annulus or other shell rings were evident after the disturbance ring (Fig. 2*a*).

Internal annuli differed markedly in appearance from internal disturbance rings. Annuli were broader, more diffuse, and lighter in colour than disturbance rings (Figs. 3–5) and were continuous from the shell exterior to the umbonal region. Annuli were widest and most diffuse in thin-shelled or fast-growing species (e.g., *Lampsilis* spp., *Leptodea fragilis*, *Potamilus purpuratus*, *Quadrula quadrula*; see Fig. 5) and on younger specimens (<5 years) of slower-growing species (e.g., *Fusconaia cerina*, *Quadrula asperata*, and *Quadrula pustulosa*). In contrast with disturbance rings, internal annuli were not associated with a disruption of the continuity of the shell surface and prismatic layer but often had a distinctive appearance where they passed through the prismatic layer. In many specimens, annuli curved abruptly within the prismatic layer and were usually deflected toward the ventral shell margin, resembling a small hook or claw (Fig. 3*c*) and were often surrounded by a clear halo (Figs. 7*b* and 8); however, these features were difficult to discern or were apparently absent in some specimens (e.g., Figs. 5*a* and 6). In some cases, an annulus was represented in the vicinity of the shell surface by a closely spaced double ring that coalesced into a single ring within the interior of the shell (Fig. 7*a*). Double annuli were rare in all species except *Obliquaria reflexa*. In *Obliquaria reflexa*, annuli were often represented by double or even multiple rings near the shell surface (Fig. 7*b*), especially in younger individuals, but, as in other species, these rings always coalesced into a single ring within the shell. No species other than *Obliquaria reflexa* had multiple annuli.

In many specimens, an external annulus was also visible at the point where the internal annulus exited the shell, but

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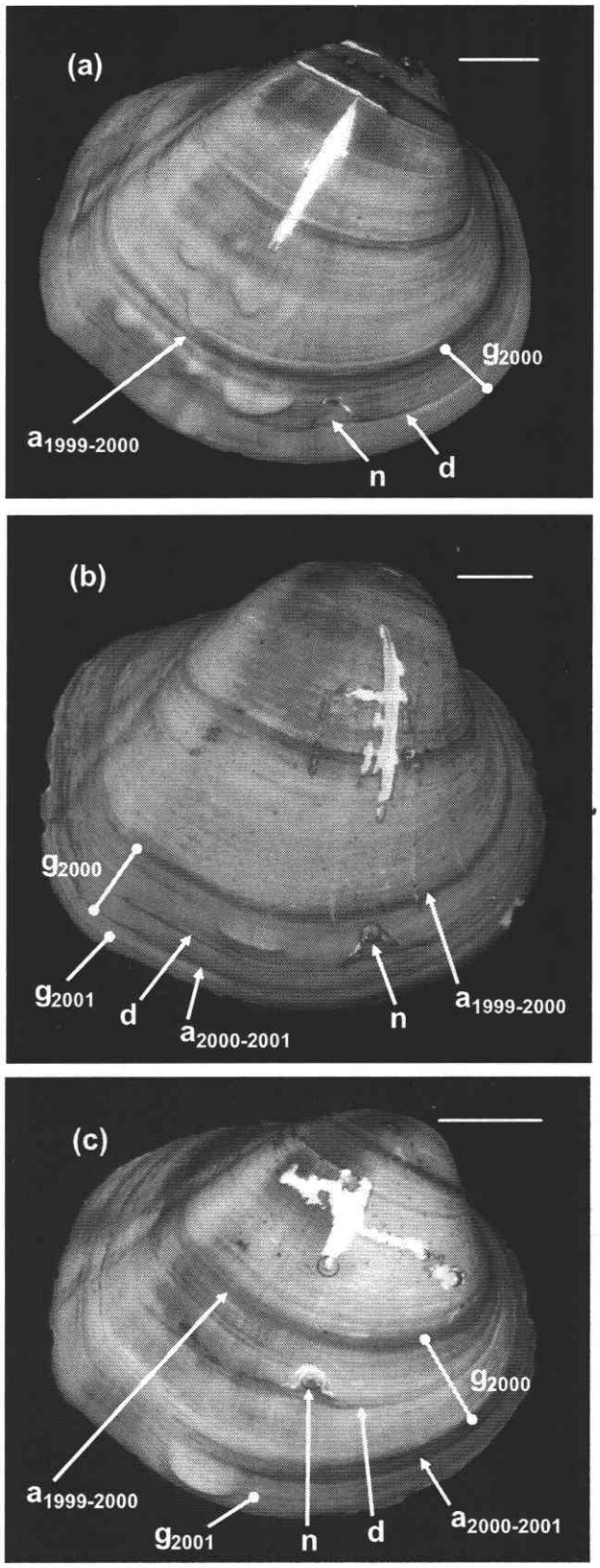

Fig. 2. Shells of *Quadrula pustulosa*, Little Tallahatchie River, Mississippi, showing growth patterns produced by notching the shell margin in August 2000. (*a*) Individual retrieved October 2000 (length and age at time of final collection: 30.1 mm, 3 years). (*b* and *c*) Individuals retrieved in August 2001. Specimen lengths and ages: (*b*) 31.3 mm, 4 years; (*c*) 23.7 mm, 3 years. n, notch; d, disturbance ring and location of shell margin at time of notching; a_x, annulus for year *x*; g_x, growth in year *x*. Long white marks perpendicular to growth rings are shallow grooves filed in the shells to facilitate quick recognition of study animals in the field. Scale bars = 5 mm.

shells often lacked unambiguous external annuli. External annuli were clearest and usually unambiguous on younger shells that were experiencing rapid growth (Fig. 2). On older shells experiencing slow growth, external annuli were usually present but were so tightly crowded that they were not interpretable.

We were unable to confirm deposition of an annulus for 12 specimens (both rivers, six species, Table 1). Of these, nine were older specimens (>19 years old) in which we could not differentiate potential annuli from disturbance rings because of low growth rates and consequent tightly crowded shell rings. However, not all older specimens were uninterpretable. Using the characters described above, we were able to distinguish annuli from disturbance rings in many older specimens of multiple species (e.g., Fig. 6). Only three younger specimens (one each: *Elliptio arca*, *Fusconaia cerina*, and *Quadrula asperata*; all from Sipsey River) deviated from our hypothesis of annulus production. These specimens each produced a clear disturbance mark in response to notching and deposited shell growth beyond the disturbance, but we could not detect an annulus or any other shell ring subsequent to the disturbance ring nor could we determine when the postdisturbance shell growth was produced. All three of these specimens were from the same tub and were the only individuals recovered from the tub, suggesting that anomalous conditions existed in the tub at some point during the experiment and were sufficient to cause abnormal growth patterns.

Validation of annuli in 2003–2005

Removal of mussels from the substrate and affixing tags to the shell consistently resulted in production of internal and external disturbance rings in both the Little Tallahatchie and St. Francis rivers (Figs. 4*b*, 5, 7–9). Even when using this less invasive marking procedure, disturbance rings were produced that were indistinguishable from disturbance rings produced by notching in 2000–2001 (e.g., Figs. 3 and 4*a*). As for notching, the degree of growth disturbance varied widely among individuals but in many cases, handling and tagging produced severe misalignment and shell margin damage (Figs. 4*b* and 5).

In 2004, the hypothesis of annual ring production was validated in both rivers, for all recovered species, and in 95% of tagged specimens that we examined (n = 133, Table 1). In all validated specimens, a disturbance ring was produced after initial collection and handling, shell growth resumed in 2003 followed by deposition of a single internal winter annulus, and additional shell growth occurred in 2004 prior to final collection (e.g., Figs. 4*b* and 5). Annuli were characterized by the same features as annuli produced by specimens in 2000–2001.

In 2004, we were unable to confirm deposition of an annulus for seven specimens (both rivers, two species, Table 1). Of these, six were older specimens (*Quadrula*

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Fig. 3. Shell thin sections showing growth patterns caused by notching the shell margin in August 2000 and retrieving specimens in August 2001, Sipsey River, Alabama. Specimen lengths and ages: (*a*) *Elliptio arca*, 59.7 mm, 5 years; (*b*) *Obliquaria reflexa*, 44.4 mm, 10 years; (*c*) *Quadrula asperata*, 43.3 mm, 13 years. Note discontinuous disturbance rings on all three specimens. d, disturbance ring and location of shell margin at time of notching; a_x, annulus for year *x*; g_x, growth in year *x*; ia, intra-annual rings; n, nacreous shell layer; p, prismatic layer. Scale bars = 1 mm.

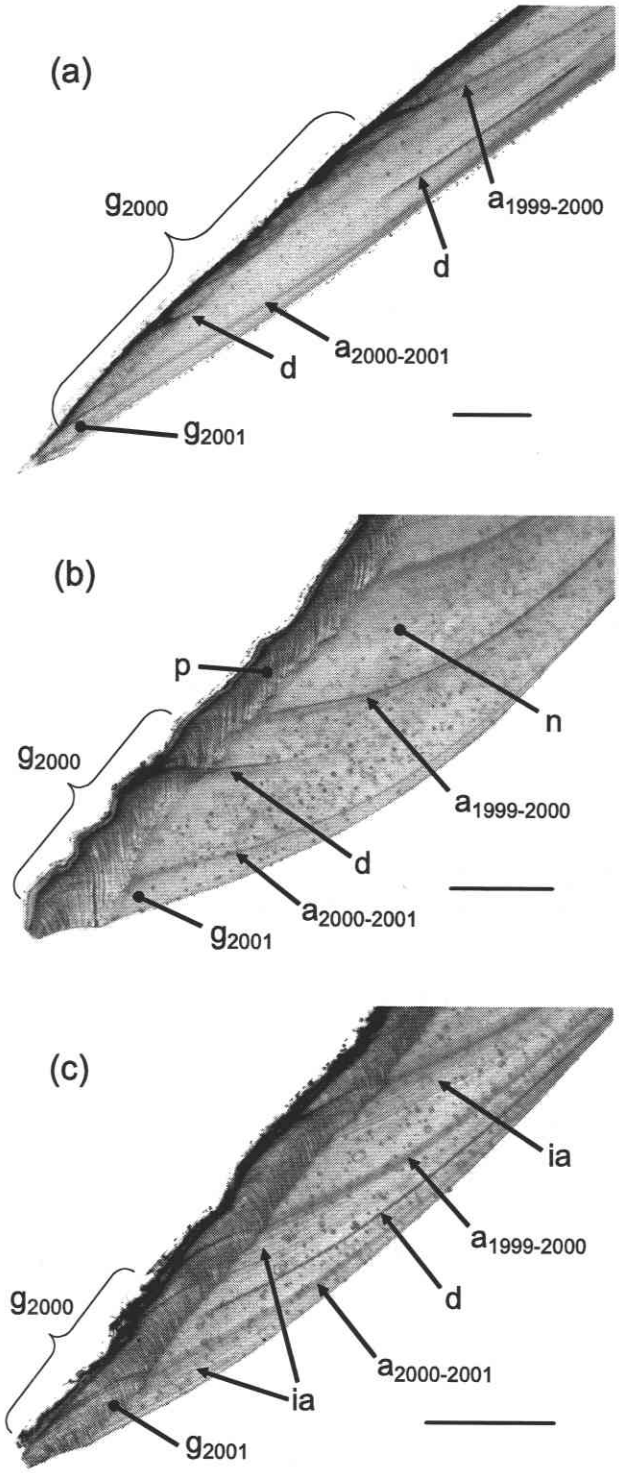
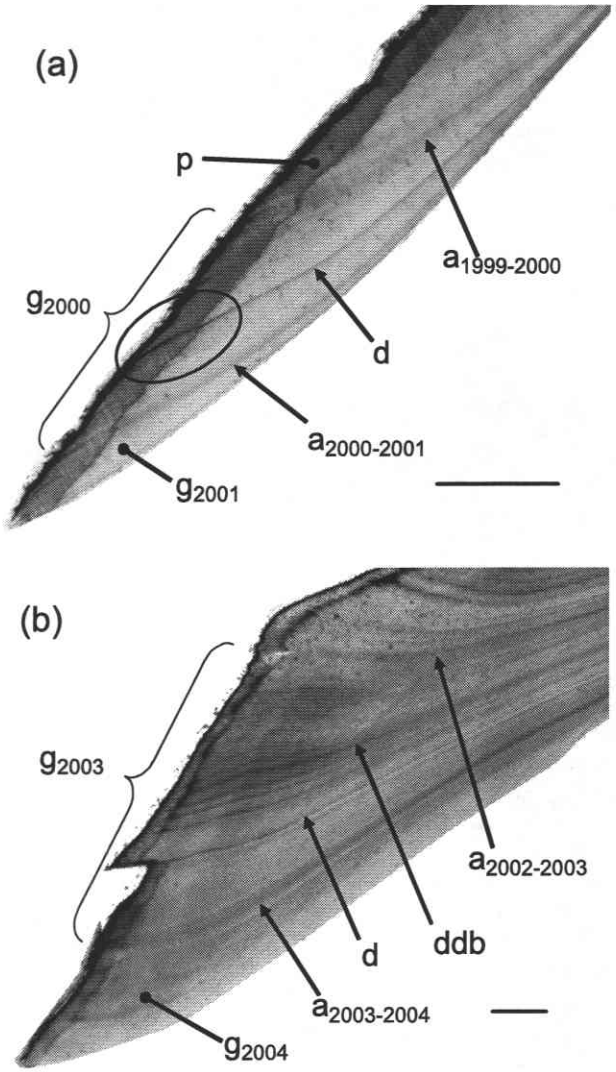


Fig. 4. Shell thin sections showing growth patterns caused by (*a*) notching the shell margin in August 2000 and retrieving specimen in August 2001, Sipsey River, Alabama, or (*b*) tagging specimen in August 2003 and retrieving in August 2004, Little Tallahatchie River, Mississippi. Ellipse on panel (*a*) highlights minor misalignment of prismatic layer after disturbance. In panel (*b*), note severe misalignment of shell growth after disturbance. Specimen lengths and ages: (*a*) *Fusconaia cerina*, 42.3 mm, age = 10 years; (*b*) *Obliquaria reflexa*, 51.2 mm, 7 years. d, disturbance ring and location of shell margin at time of notching or tagging; a_x, annulus for year *x*; g_x, growth in year *x*; ddb, diffuse dark band; p, prismatic layer. Scale bars = 1 mm.



pustulosa) with tightly crowded shell rings, similar to unvalidated specimens from 2000–2001. No specimens with interpretable thin sections deviated from our hypothesis of annulus production. However, in one specimen (*Potamilus purpuratus*, St. Francis River), we were unable to detect a disturbance ring produced in response to handling. This specimen had well-defined shell rings that were indistinguishable from annuli in other validated specimens of *Potamilus purpuratus*, but because the specimen did not grow measurably between initial and final collection, disturbance

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Fig. 5. Shell thin sections showing growth patterns and shell margin damage produced by tagging animals in August 2003 and retrieving in August 2004, St. Francis River, Arkansas. Specimen lengths and ages: (*a*) *Quadrula quadrula*, 77.0 mm, 14 years; (*b*) *Leptodea fragilis*, 106.7 mm, 5 years. d, disturbance ring and location of shell margin at time of tagging; a_x, annulus for year *x*; g_x, growth in year *x*. Scale bars = 1 mm.

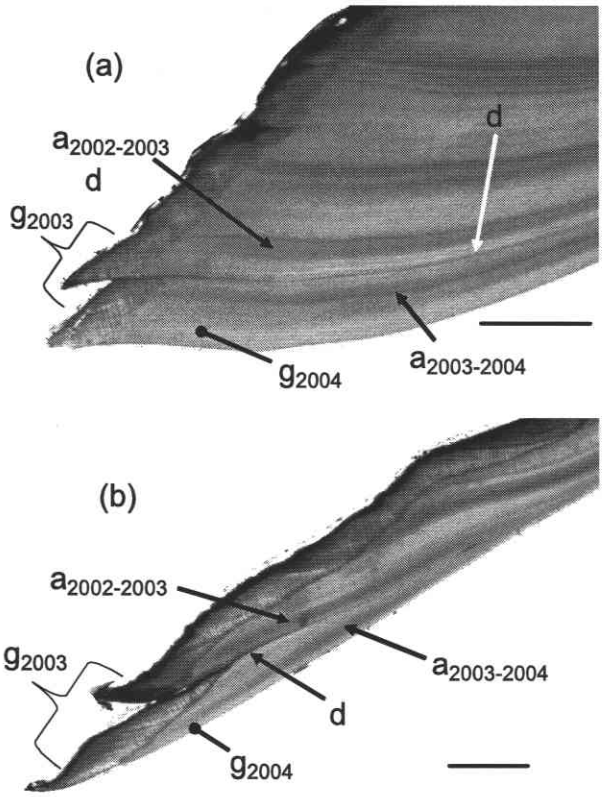
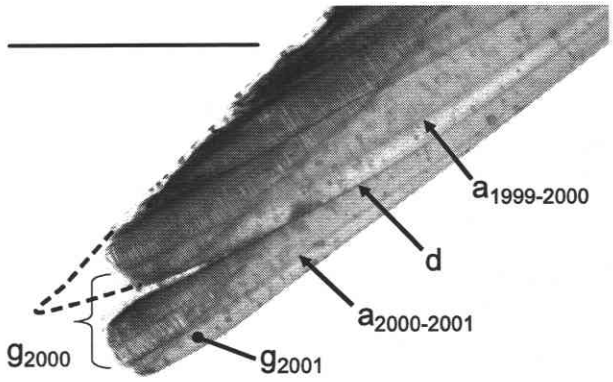


Fig. 6. Shell thin section of *Fusconaia cerina* showing growth patterns caused by notching the shell margin in August 2000 and retrieving specimen in August 2001, Sipsey River, Alabama (length = 55.3 mm, age = 25 years). Broken line shows approximate extent of shell at time of notching. d, disturbance ring and location of shell margin at time of notching; a_x, annulus for year *x*; g_x, growth in year *x*. Scale bar = 1 mm.



rings and annuli deposited during the experiment may have been superimposed, and we were unable to confirm the timing of shell ring deposition.

In 2005, the hypothesis of annual ring production was validated in both rivers, for all recovered species, and in 97% of recovered tagged specimens (n = 74, Table 1). Disturbance rings and annuli in 2005 specimens were identical in appearance to those shell features in specimens from 2004 and 2001. We were unable to confirm deposition of an annulus for only a single specimen in each river. In the Little Tallahatchie River, we could not validate an old specimen of *Quadrula pustulosa* with tightly crowded shell rings. In the St. Francis River, a large specimen of *Lampsilis teres* did not grow after initial marking in 2003; consequently, annuli and other shell features, if present, were essentially superimposed and not interpretable. In all but two validated specimens from the Little Tallahatchie River, we were able to correctly deduce the collection history of the specimen based on our identification of disturbance rings and annuli (Fig. 8), as confirmed by later comparison with reference points on the shell. The two specimens whose collection history we deduced incorrectly were collected in 2003 but not recovered until 2005; both specimens produced a disturbance ring in 2003 and two annuli as expected, but we also identified a faint disturbance ring deposited in 2004, even though these specimens were not handled by us in 2004. In these specimens, the 2004 disturbance ring may have been caused by a natural growth interruption or by our collecting activities in the vicinity.

Non-annual shell rings

In addition to annuli and disturbance rings associated with handling and marking, we observed apparent natural disturbance rings, intra-annular rings, and diffuse dark bands. Disturbance rings, identical to those caused by handling during our study, were seen in several specimens in portions of the shell deposited prior to our study. Natural disturbance rings occurred not uncommonly but irregularly; we observed no obvious pattern in their occurrence among species, rivers, or years, but we made no systematic attempt to detect such patterns.

Numerous and closely spaced intra-annular rings were present in nearly all shells, but they were usually indistinct and difficult to discern clearly. Shells with relatively distinct intra-annual rings had a minimum of about 20 rings between each pair of annuli, but even in these specimens it was impossible to count all intra-annular rings reliably. When visible, intra-annular rings were continuous throughout the shell and exited through the prismatic layer, similar to annuli, but intra-annular rings were always fainter than annuli and in many cases appeared only as faint shadows. Even in specimens with more distinct intra-annular rings, intra-annular rings were clearly distinguishable from annuli (Fig. 3*c*).

Diffuse dark bands of varying widths appeared in many specimens and were consistently distinguishable from annuli and disturbance rings. Diffuse dark bands were present only within the interior of the shell and did not continue through the prismatic layer to the shell surface and so did not produce the distinctive hook associated with annuli where they exited the shell (Figs. 4*b* and 7*a*). Within a single specimen, diffuse dark bands and annuli usually differed in colour; diffuse dark bands were darker than annuli in some specimens, but lighter in others. Diffuse dark bands were often discontinuous throughout the interior of the shell, but in other cases extended throughout the shell with the exception of the prismatic layer. Diffuse dark bands were most common and

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Table 1. Validation of the hypothesis of annual shell ring production in 17 species of freshwater mussels from three rivers in the southeastern United States.

	Number examined (number validated)					
	Sipsey	Little Tallahatchie			St. Francis	
Species	2001	2001	2004	2005	2004	2005
Amblema plicata	—	12 (12)	12 (12)	2 (2)	1 (1)	—
Elliptio arca	10 (9)	—	—	—	—	—
Fusconaia cerina	33 (27)	—	—	—	—	—
Fusconaia flava	—	—	—	—	1 (1)	—
Lampsilis cardium	—	—	1 (1)	—	—	—
Lampsilis ornata	3 (3)	—	—	—	—	—
Lampsilis teres	—	2 (2)	—	—	7 (7)	2 (1)
Leptodea fragilis	—	1 (1)	—	—	6 (6)	1 (1)
Obliquaria reflexa	4 (4)	9 (9)	17 (17)	4 (4)	9 (9)	—
Plectomerus dombeyanus	—	—	—	1 (1)	—	—
Potamilus purpuratus	—	—	—	—	9 (8)	1 (1)
Pyganodon grandis	—	—	1 (1)	—	—	—
Quadrula asperata	26 (23)	—	—	—	—	—
Quadrula pustulosa	—	40 (39)	57 (51)	59 (58)	—	—
Quadrula quadrula	—	—	1 (1)	1 (1)	10 (10)	2 (2)
Quadrula rumphiana	7 (6)	—	—	—	—	—
Tritogonia verrucosa	10 (10)	—	1 (1)	1 (1)	—	—
Totals	93 (82)	64 (63)	90 (84)	68 (67)	43 (42)	6 (5)

often ubiquitous in specimens less than about 5 years old for all species, especially *Amblema plicata* and *Obliquaria reflexa*. In young specimens, there were often multiple, irregularly spaced diffuse dark bands between each pair of annuli (Fig. 4*b*). In older specimens, diffuse dark bands usually appeared singly and were rare, with a single exception. In 2004 at the Little Tallahatchie River, numerous individuals from multiple species deposited a single diffuse dark band about midway between the onset of growth in spring and collection in midsummer (Fig. 7*a*). This diffuse dark band was seen in 2004 in 72% of validated *Quadrula pustulosa* and in *Amblema plicata*, *Obliquaria reflexa*, and *Tritogonia verrucosa*.

Effects of handling on growth

Most tagged individuals grew between initial marking and final collection. Young individuals grew rapidly but in older specimens, 1-year growth increments were small to nearly imperceptible. In some specimens, although considerable new shell material was deposited after initial collection, shell margin damage associated with handling resulted in little or no increase in length (e.g., Fig. 5*a*), or, more rarely, a decrease in size (Fig. 6). We observed a decrease in size between 2003 and 2004 in four species, but this phenomenon occurred only in large individuals with slow growth rates. In the Little Tallahatchie River, 28 specimens of *Quadrula pustulosa* (15% of total tagged individuals) showed unequivocal evidence of a decrease in size from 2003 to 2004, when taking into account the limits of our measurement precision (Fig. 10, see Materials and methods). All specimens that decreased in size were >47.0 mm length in 2003; the maximum decrease was 2.9 ± 0.1 mm, but most specimens decreased by <1.0 mm (mean = 0.6; Fig. 10). We observed a decrease in size in the Little Tallahatchie River for only one other individual (*Pyganodon grandis*, from 114.4 to 113.7 mm). In the St. Francis River, five specimens of *Quadrula quadrula* (50% of total) decreased in length (mean decrease = 1.0 mm), and one specimen of *Potamilus purpuratus* decreased from 103.4 to 98.9 mm.

Observed growth from 2003 to 2004 was similar to growth predicted by shell annuli. For *Obliquaria reflexa* and *Quadrula pustulosa*, the regression line for growth predicted by annuli fell within the 95% prediction interval around observed growth throughout the entire range of shell length (Fig. 11). The predicted growth line for *Amblema plicata* based on annuli fell slightly above the observed 95% prediction interval for specimens less than about 50 mm length, but was encompassed by the interval for larger specimens (Fig. 11).

Despite the general similarity of observed and predicted growth, mean observed 2004 length was significantly lower than predicted by annuli for all three thick-shelled species (Table 2), but the magnitude of difference was small. For all three species, mean observed 2004 length was 1.4%–5.0% less than predicted 2004 length (Table 2). For thin-shelled species, small sample sizes limited our ability to make firm conclusions about growth. The difference between observed and predicted growth of *Lampsilis teres* and male *Potamilus purpuratus* appeared of similar magnitude or slightly greater than thick-shelled species, but observed growth of *Leptodea fragilis* appeared to deviate from predicted growth by a wider margin (Table 2). We also observed little postmarking growth in *Lampsilis ornata* and *Pyganodon grandis*, two other thin-shelled species that typically have high growth rates (W. Haag, unpublished data), but recovered few individuals of these species.

Repeated handling over 2 years resulted in an accrual of growth reduction in *Quadrula pustulosa* (Fig. 12). Final 2005 length was significantly lower for individuals that were handled in both 2003 and in 2004 than for individuals that

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growth and shell damage caused by handling. This conclusion is supported by two lines of evidence from this study and by other published studies. First, the pervasive formation of disturbance rings in all of our study specimens shows that handling consistently results in at least temporary disruption of growth. Disruption appears to be minor and of brief duration in most specimens, judging by the low degree of shell damage and resumption of growth after handling, but some specimens sustained serious shell damage and grew little or even decreased in size after handling. Second, mussels that were handled twice showed significantly lower growth than mussels that were handled only once during the same time period, showing that repeated handling results in an accrual of growth impacts. Because bivalves deposit new shell material at the mantle edge, growth is interrupted when the mantle is withdrawn from the shell margin in response to handling or other acute disturbance (Coker et al. 1921; Richardson et al. 1980; Mutvei and Westermark 2001). *Anodonta anatina* kept in aquaria formed a disturbance ring each time they were handled (Negus 1966). Further, reestablishment of the mantle–shell margin connection after disturbance often occurs at a position offset from the original plane of growth, resulting in shell margin damage and an apparent decrease in growth (Richardson 1989; this study). Removal of mussels from the substrate nearly always results in withdrawal of the mantle to some extent. Therefore, even careful handling should be expected to result in a temporary interruption of shell deposition, potential shell damage, and in many cases, measurable reductions in growth or decreases in size.

The sensitivity of mussels to handling has important implications for design of future growth studies and for the interpretation of some previous studies. Mark–recapture studies are used commonly for estimating growth in bivalves, including freshwater mussels (e.g., Bailey and Green 1988). Even though, as in our study, errors introduced by handling stress may be small, mark–recapture studies should acknowledge the potential for underestimating growth to some degree. Handling effects could be reduced by delaying recapture for 2 years after initial collection, therefore leaving animals unmolested for a full year, and by avoiding repeated handling during the course of the study. Previously, growth rates of mussels from a mark–recapture experiment that were lower than predicted by internal shell rings have been interpreted as evidence that internal rings are not produced annually (Kesler and Downing 1997). Because that study involved collecting and measuring marked animals every year for several years, it is likely that the lower growth rate of marked animals was an artifact of repeated handling. Although the study examined potential marking effects by simultaneously measuring growth in unmarked control animals, controls were also handled and measured and therefore experienced growth disruption similar to marked animals.

In another mark–recapture study involving repeated handling of marked animals, length of many specimens decreased during the study, leading to the conclusions that shell loss is a common phenomenon in freshwater mussels and their shells are therefore of questionable usefulness as long-term records of growth (Downing et al. 1992; Downing and Downing 1993). However, as in our study, these decreases in shell length are better explained by chronic growth interruption and shell margin damage associated with repeated handling and not as evidence that extensive dissolution of the shell margin occurs frequently in nature. Shell loss we observed as a result of handling was restricted to the distal edge of the periostracum and prismatic layer and did not involve loss or erasure of growth information contained within the nacreous shell layers. In older shells, erosion of the umbonal region can result in loss of the growth record for the first few years of life, but these losses can be accounted for using length-at-age data (Hastie et al. 2000). For these reasons, the value of mussel shells as long-term records of growth and environmental conditions is becoming well accepted (Carell et al. 1987; Nyström et al. 1996; Mutvei and Westermark 2001).

The sensitivity of mussels to handling also raises important conservation questions. Because mussels must be removed from the substrate for identification and measurement, nearly all routine mussel surveys and monitoring protocols have the potential for causing minor shell damage and measurable reductions in growth. We emphasize that although reductions in growth due to handling are detectable statistically, these differences are of very small magnitude. Further, substantial shell damage occurs in a minority of handled specimens, and most specimens sustain only minor or no damage. These impacts likely pose little threat to mussel survivorship or fitness. Although our study was not designed to measure survivorship, we recovered a high percentage of live mussels in 2004 and 2005 combined that were tagged in 2003 (*Amblema plicata* 92%, *Obliquaria reflexa* 96%, *Quadrula pustulosa* 82%) at the impounded Little Tallahatchie River site, all of which were found in the normal siphoning position just under the substrate surface. We also found no dead, tagged shells, and because of the very slow current at the site, shells could not have been transported. Lower recovery rates at the other two sites are attributable to the more dynamic nature of these unimpounded streams and provide no useful information about survivorship. A wide variety of other studies have shown high survivorship in mark–recapture studies (e.g., Neves and Moyer 1988; Berg et al. 1995; Kesler and Downing 1997), further supporting the notion that careful handling does not result in increased mortality. Nevertheless, repeated handling results in an accrual of growth impacts that could ultimately have negative effects on mussel survival or fitness. These potential impacts warrant further evaluation, but at this time we see no need to consider restrictions of routine survey and monitoring activities based on the potential effects of handling on growth.

In conclusion, this study confirms the long-held assumption that freshwater mussels deposit annual shell rings similar to those of marine bivalves and to rings in hard structures in many other organisms. Internal annual rings in mussel shell thin sections can be used to derive robust estimates of age, growth, and longevity. Although annuli deposited on the external shell surface are unambiguous and reliable in young specimens, external rings are not consistently reliable indicators of age in most situations. Other types of internal shell rings, including disturbance rings and intra-annual rings, can be reliably distinguished from annuli and hold great potential for providing an array of ecological information. Our confirmation of annulus production across multiple species,

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rivers, and years, combined with similar observations from previous studies, suggests that formation of annual rings is a pervasive phenomenon in freshwater mussels in temperate regions.

Despite the generality of annulus production by freshwater mussels, validation of putative annual rings remains an essential prerequisite for any growth study. Differentiation of annuli from non-annual shell rings is based on qualitative characters that can vary among mussel species and are therefore context-specific. Even in well-studied organisms, variations in growth and ring formation among species, habitats, and age classes can lead to serious errors when interpreting unvalidated indicators of age (Beamish and McFarlane 1983). For fishes, comparison of growth rings with those from hatchery-raised specimens of known age can correct many errors in interpretation (Buckmeier 2002), but this technique is currently not widely applicable for freshwater mussels. Therefore, a primary benefit of validation of mussel shell rings is providing a comparative set of shell features from specimens with a known growth history, specific to a particular species at a particular locality. Such a reference will aid greatly in accurately identifying annuli, disturbance rings, and other shell features. For example, without conducting this validation study, the closely spaced multiple annuli deposited in a single year by *Obliquaria reflexa* would likely have been misinterpreted as representing several consecutive years of low growth. For all species, the primary difficulty we encountered was validating growth rings on old specimens with crowded rings. In future studies, we recommend using a more precise growth marker (e.g., Kaehler and McQuaid 1999; Fujikura et al. 2003) that will facilitate validation of shell rings regardless of the age of the specimen.

The formation of a disturbance ring by nearly all of our study specimens shows that freshwater mussels are extremely sensitive to handling. Although careful handling is not likely to result in increased mortality, any handling that requires removal of the animal from the substrate can result in reduced growth and will likely result in production of a disturbance ring. Some of our observations suggested that thin-shelled species may be more sensitive to handling than heavy-shelled species, but we were not able to evaluate this observation rigorously. Mark–recapture studies designed to estimate growth or examine periodicity of shell rings must take into account the likelihood of growth disruptions associated with handling. For this reason, examination of validated internal shell annuli will provide the most accurate estimates of age and growth in freshwater mussels.

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