Relative RNA Content as a Measure of Condition in Larval and Juvenile Fish

Iain M. SuthersA, Jennifer J. ClearyA,B, Stephen C. BattagleneB,C and Rachael EvansA

ASchool of Biological Sciences, University of New South Wales, Sydney, NSW 2052, Australia.
BNew South Wales Fisheries, Port Stephens Research Centre, Salamander Bay, NSW 2315, Australia.
CPresent address: ICLARM, PO Box 879, Honolulu, Solomon Islands.

Abstract. The RNA:DNA ratio for first-feeding larvae (12 days after hatching, 4–6 mm standard length, SL) of Australian bass (Pseudechidnus macrochirra) exposed to four different feeding regimes over 8 days was found to be insensitive to the level of starvation. An alternative condition index based on residuals derived from the regression of RNA on SL showed significant differences over the course of the experiment; these reflected the four feeding treatments. Field collections of juvenile monomacchids (Pseudomaculatus etioschis, 10–30 mm SL) from a local estuary revealed no significant difference in RNA:DNA ratio at three sites over six weeks. A residual-based index (RNA on dry weight) showed parallel fluctuations at all sites; they were positively correlated with water temperature. The RNA:DNA ratio depends on the difference in fluorescence between total nuclear RNA (using thiazole orange) and DNA (using Hoechst 33258) to calculate RNA, as there is no RNA-specific fluorescent dye. The numerator is thus dependent on the denominator, and measurement error may be compounded in the ratio, exacerbating potential variability in the index. Ratios may also be variably correlated with age or size and consequently may erroneously indicate condition or growth in larger and faster-growing fish.

Introduction

Accurate assessment of larval condition or health has widespread application in the fields of fisheries management, aquaculture and fish ecology, as an indicator of sub-lethal effects. Many indices have been used to determine nutritional condition in larval fish, involving morphological (e.g. Ehrlich et al. 1976; Wright and Martin 1985), histological (e.g. Ehrlich et al. 1976; Thörnaker 1986; Martin and Wright 1987; Bisbal and Bengston 1995) and biochemical techniques (e.g. Buckle 1984; review in Ferron and Leggett 1994).

The RNA:DNA ratio is based on the assumption that the concentration of RNA changes as a function of the amount of protein synthesis occurring in the cell (Ballow 1970; Regnault and Loquet 1974; Buckle 1981, 1984). The amount of DNA in a cell is relatively constant for all somatic cells for a given species (Buckle 1980, 1984), and so indicates the number of cells in an organism. Therefore, the RNA:DNA ratio is considered a measure of protein synthetic activity per cell (Raae et al. 1988). Larvae in good condition tend to have a higher RNA:DNA ratio than those in poorer condition (e.g. Robinson and Ware 1988; Clemmesen 1990). It is reported that the ratio can respond to changes in environmental conditions within 1–3 days (Martin and Wright 1987), and the ratio has been used to give a measure of instantaneous growth in the field, avoiding periodic measurements (Buckle 1981; Clarke et al. 1989).

Recent studies have advocated caution in the use of RNA:DNA ratios on the basis of techniques (extractions methods, McGurk and Kussoc 1992) type and quality of RNA and DNA standards, Caldrocare and Buckle 1991, endogenous fluorescence in juvenile fish, Camino and Caldrocare 1994, or lack of sensitivity (Bergeron et al. 1991, Richard et al. 1991; Matbers et al. 1994). Other workers have advocated caution in the use of ratios in removal of the aleometric effect of size (e.g. Reze 1985; Packard and Boardman 1987), but rarely in the context of a widely used technique such as the RNA:DNA ratio (Hovenkamp and Wiele 1991). Here, the numerator and/or the denominator can vary to produce a similar ratio.

The present study examined the ability of the RNA:DNA ratio to assess nutritional condition on the basis of whole homogenates of larvae from controlled and field conditions. Condition of Australian bass larvae (Macquaria novemaculata) was altered in four feeding treatments in the laboratory, and recently settled monomacchids (Pseudomaculatus etioschis) were studied in a large estuary. The aims were firstly to compare the ratio to a residual-based index derived from RNA content and an independently determined variable such as standard length or dry weight and secondly to evaluate a relatively new and
sensitive dye, thiazole orange, by means of a simplified extraction technique (after Berdächt and Dorhöf 1991).

Materials and Methods

Laboratory Study

Specimens of adult M. nematocystis, a catadromous periclidid, were captured from Seamen Weir, William River, New South Wales, in July 1992, and twelve pairs were injected to spawn. Four 60 L aerated flow-through glass aquaria were set up in a room at constant temperature (27±1°C) with a 12:12 light-dark cycle. Water temperatures varied between 19°C and 19°C, and salinities varied between 35 and 36.

On the first day after hatching (Day 0), larvae were stocked into four tanks at a density of 750 to 250 larvae. During final absorption of the yolk sac and oil globule, larvae were fed retinyl acetate (aqueous solution) once daily at 10 mL L−1. (Talbot et al. 1990) from Bay 8 until most of the larvae were feeding (Day 12). After Day 12, the feeding regimes were as follows: (1) PF, fully fed; (2) PF, fully fed; (3) PF, fully fed; (4) PF, fully fed. After that, 15 ml L−1 per day was fed. After Day 15, 10 ml L−1 was fed per day. All feedings were done at 21:00. From Day 15 to 15 ml L−1, 5 ml L−1 was fed per day. From Day 16 to 15 ml L−1 was fed. The experiment continued until 100% mortality occurred in the FS tank on Day 20. All the data were treated using a one-way ANOVA for a possible day before feeding. For statistical analysis, there was only one tank per treatment, and consequently our conclusions are tank-specific and are assumed to be in response to the starvation treatment. Standard lengths (SLs) were measured using a binocular microscope before the individual larvae were stored in liquid nitrogen for total nucleic acids (TNA) and DNA analysis. Indices were measured across treatments on Days 14, 16, and 20 with separate factor analyses of variance (ANOVA) (significance level at P < 0.05). There was no significant heterogeneity of the data (Bartlett's test). Treatments were compared within each day by Tukey's test adjusted for unbalanced data.

Field Study

Boat Bay is a large (56 km2) embayment in south-east Sydney, New South Wales, fed by the Georges and Kitchen rivers. The bay is shallow, with a maximum depth of 10 m in the dredged shipping channel. Three shallow seagrass and sand sites, separated by 6–12 km, were sampled with an epibenthic sled (40 × 40 cm; mesh size, 500 μm mesh in 1, 1.8, and 2.7 June and 17 July 1993). At each site, three 5-m transects were conducted over a broad area to avoid sampled regions, with water temperature being recorded at the surface and the bottom at the beginning and end of sampling. The dominant species was the dusky leathery/jacket, Paramonostoma setiferum (Mansoniidae). Modifications in the stratification of RNA and DNA used in the laboratory study are shown in Fig. 1, with the major differences being to freeze-dry the larvae before they were homogenized. Ratio and residual indices were calculated across treatments on each sampling date, with a separate ANOVA for each tissue because 1 of 6 sites did not occur at all sites on all dates. Then, indices were compared at site 4 across the four sites in a separate ANOVA.

Analytical Procedure

The technique involved the use of thiazole orange (TO) to determine DNA-ST vs. concentration of DNA-ST vs. concentration of DNA-St (Sigma), in conjunction with Tris-Cl buffer. 1 L, 1 ml, adapted from the double fluorometric method for phytoplankton developed by Bonnabel and Ernö 1991. Larvae were thoroughly homogenized by hand in 10 mL, before freezing, homogenizing glass tubes with 10 mL, filtered (Tris-Cl buffer), and then sonicated for 30 s before being combined with the dye. The concentrations of DNA and TO used (Fig. 1) were appropriate for the larvae (Cary 1992) and assayed (Levov 1993) used in this study. All materials were maintained on crushed ice.

Standard nucleic acid solutions were made from single-stranded calf-thymus DNA Type I (Bioscientific, DB399) and baker’s yeast RNA Type III (Bioscientific RT125). These standard curves were run daily (DNA-HO; DNA-TA; RNA-TA). RNA and DNA concentrations were calculated from the three standard curves and the fluorescence values obtained for each sample. A Perkin Elmer fluorescence spectrophotometer measured the nucleic acid-type dye complex fluorescence for the larval fish and standard curves. This machine was run on the DC mode with the slit widths both of the excitation and emission wavelengths set to 10 nm. Spike recoveries of RNA and DNA usually averaged 90% and 95% respectively. The fluorescence procedure for the field catch fish had to significant effect on RNA or DNA content of filterable homogenate.

Results

Laboratory Study

The size of the larvae ranged from approximately 4.3 mm to 5.6 mm during the course of the experiment (Fig 2). Fully fed fish grew on average 0.1 mm day−1 from Day 13 to 20, while fully starved grew on average 0.01 mm day−1. The S1 and S3 fish were intermediate, growing 0.07 and 0.04 mm day−1 respectively (Fig. 2). FF larvae were significantly larger than S1 and S3 larvae on Day 20, but not on Day 18, 16 or 14.

The amount of RNA and DNA increased with SL, with DNA showing a curvilinear response to SL (Fig. 3). From
the relationship of ln(RNA+1) or ln(SL), residual indices were calculated. Residual plots show no remaining trends or non-linearity.

The mean RNA:DNA ratios for each of the four treatments for each day show no systematic trend across days or among the treatments (Fig. 4a).

The mean residual condition (RNA-SL, Fig. 4b) shows a trend of enhanced condition for the FF and S1 treatments over S3 and FS. On Day 16 and Day 18 the mean FF residual indices were significantly greater than FS and S3 (Tukey's P < 0.02), but FF was never significantly different to S1. By Day 20, S3 and FS indices increased and none of the treatments were significantly different (Fig. 4c). The FS treatment showed a rapid rise in apparent condition 2 days before death, due to a relative increase in RNA with respect to SL.

**Field Study**

Between 6 and 24 Parancanthurus ocellaris were caught at each site on each date, ranging between 10 and 42 mm SL (1-230 mg dry weight), although fish >80 mm SL (120 mg) were excluded from biochemical analysis (n = 110

**Fig. 3.** Relationship of (a) RNA (μg) and (b) DNA (μg) on standard length (mm, SL) of *M. sexocelaris*; FF (0), fully fed through entire experiment; S1 (3), starved on Day 13 then fed; S3 (1), starved on Days 13, 14 and 15 then fed; and FS (0), fully starved. ln(RNA + 1) = 1.77 (± 0.36 * ln(SL)), (n = 203), r² = 0.27; ln(DNA + 1) = 1.99 (± 0.99 * ln(SL)), (n = 203), r² = 0.42.

**Fig. 4.** Change in (a) average RNA:DNA ratio and (b) average residual of ln(RNA + 1) vs. SL of *M. sexocelaris* larvae over the four feeding treatments. FF (0), fully fed through entire experiment; S3 (1), starved on Day 13 then fed; S1 (3), starved on Days 13, 14 and 15 then fed; and FS (0), fully starved. Solid bars show periods of starvation for S1 and S3. Error bars are standard error.
for nucleic acid analysis. If it is assumed that the same population was being sampled, growth at Site 1 was slight (0.11 mm day\(^{-1}\)) and then declined. At Sites 2 and 3 average SL increased at approximately 0.40 and 0.37 mm day\(^{-1}\) respectively, but by mid July growth declined or ceased (0.18 and -0.04 mm day\(^{-1}\) respectively, Fig. 5).

![Graph showing standard length (mm) over time for Sites 1, 2, and 3.](image1)

**Fig. 5.** Change in average SL of *P. ostenisii* over the three sites and four dates in 1999. Error bars are standard error. Sample size is indicated.

RNA and DNA were linearly related to dry weight (Fig. 6), with no pattern in the residuals and there was no significant difference in the slopes among sites or dates (ANOVA, \(P > 0.3\)). Consequently, a common regression was fitted to calculate individual residual condition indices. The RNA–DNA ratios showed no significant difference among sites on each date (four 1-way ANOVAs, \(P > 0.1\)) and no significant difference or trends among dates at Site 3 (Fig. 7a). The RNA–DW residuals also showed no significant differences among sites or dates (Fig. 7b) but did exhibit parallel trends across all sites. The ratio showed no causal relationship with temperature (Fig. 8a), whereas the residuals from RNA–DW were significantly correlated with water temperature (\(r^2 = 0.61\), Fig. 8b).

**Discussion**

**RNA–DNA Ratio and Residual Indices**

The RNA–DNA ratio derived from the technique in Fig. 1 proved unsuitable as a diagnostic tool of larval condition in Australian bass (Fig. 4a), despite starvation of the larvae under controlled laboratory conditions. In the field, RNA–DNA ratios of dusky leatherjacket did fluctuate synchronously at three sites over six weeks, although there was no relationship with the 4°C range in water temperature (Fig. 8a).

![Graph showing relationship of RNA (μg) and DNA (μg) on dry weight (mg) for *P. ostenisii*. Symbols as in Fig. 3. RNA = 94.39 + 2.48*DW (\(r = 0.68\), \(r^2 = 0.46\)), DNA = 48.24 + 2.26*DW (\(r = 0.62\), \(r^2 = 0.48\)).](image2)

**Fig. 6.** Relationship of (a) RNA (μg) and (b) DNA (μg) on dry weight (mg) for *P. ostenisii*. Symbols as in Fig. 3. RNA = 94.39 + 2.48*DW (\(r = 0.68\), \(r^2 = 0.46\)), DNA = 48.24 + 2.26*DW (\(r = 0.62\), \(r^2 = 0.48\)).

In contrast, the use of residual indices did reveal significant effects of starvation in the laboratory. In the field, the residuals revealed a general decline in condition on the third sampling date (27 June) at one site, and all sites exhibited parallel fluctuations over 6 weeks that were correlated with water temperature (Fig. 8b). With other biochemical techniques, however, RNA–DNA ratios are normally correlated with temperature (e.g. Buckley 1982). Although the ratio was correlated with the residual index overall (M. novaculaeua, \(r = 0.52\); *P. ostenisii, r = 0.65\), these results illustrate where possible errors in the calculation of DNA and TNA are magnified in the ratio. At RNA (the numerator) is derived from DNA (the denominator), any errors in the measurement of DNA and TNA are magnified in the final ratio (Packard and Boardman 1987; Hovenkamp and Winne 1991).

Another potential source of error arises if the ratio is correlated with site (e.g. Buckley et al. 1984; Robinson and Ware 1988; Westerman and Holt 1994; Clennemosen 1994), although we did not find the correlation of the ratio with either SL or dry weight that had been observed in other studies (e.g. Buckley et al. 1984; Buckley and Lough 1987). However, some studies have shown an allometric effect of the RNA:DNA ratio (Westerman and Holt 1988; Cantino et
Relative RNA Content in Larval and Juvenile Fish

growing, and these fish will be bigger. Nevertheless, the effect of size is still incorporated in the ratios and may confound studies with unknown (field) conditions. In the field, Robinson and Ware (1988) observed over time that larger larvae had a higher RNA/DNA ratio, but they were careful to note that generally within a day there was no significant correlation between ratio and SL. Alternatively, the ratio itself can be de-trended (e.g. Camino et al. 1991). Residuals from a simple linear regression can simply remove the effect of size and provide a powerful way around ratios because linear models can be built to incorporate the effect of temperature, for example. The ANCOVA is a complementary analysis by comparing slopes and treatments (e.g. Bailey et al., 1995), and it can provide an individual index such as a residual. Different techniques and species can differ in their yields of nucleic acid (Caldataone and Buckley 1991; McGurk and Kusser 1992), so residuals are solely relative within any one study and the intra-comparison of ratios is not valid. Similarly, the conversion of RNA/DNA ratios to daily protein growth rates using Buckley's (1984) generalization equation is not recommended without running an intercalibration (Camino and Caldataone 1994).

In the present study, because RNA is used in the calculation of RNA, standard length (e.g. Bailey et al., 1995) or dry weight were used as independent variables to standardize RNA. In juvenile fish, other components such as lipid can influence size not accounted for by the number of cells and by DNA. By using least-squares regression (rather than geometric-mean regression), we assumed that the measurement error of dry weight (or SL) is small relative to that of RNA.

The laboratory study produced a surprising result in the residual analysis for the F5 treatment, with the proportion of RNA increasing markedly two days before death (Fig. 4b). A similar trend in the RNA-DW residual was evident in an independent data set for larval wrasse (G. Caldataone, unpublished data). These larvae may be resorting to cannibalism, but such a trend is not evident in the ratio (Fig. 4a). This appears to be the result of physiological stress, and such fish are morphologically distinctive and would presumably be removed by predation in the field.

The laboratory study also presented difficulties in generating residuals, not encountered with field larvae, because the starved treatment had a smaller size range of larvae, increasing the possibility of non-homogeneity of slopes between treatments. In our analyses from the laboratory and field, the ANCOVA was used solely to ensure homogeneity of slopes, because the larvae came from samples on different dates. We assumed that slopes were similar among days. Ideally, the ANCOVA should incorporate day and treatment effects, but sample size was insufficient on each day to confidently produce a regression. The laboratory example had only one task per feeding
treatment because of logistic constraints and, although the trends observed could be tank effects, we assumed that they represented the feeding treatments.

Tbassuca Orange and RNA Residuals

Berdalat and Dorch (1991) found fluorescence yield of TO was about 20 fold that of echium bromide, and this was evident in our trials (Clark 1992). Our extraction procedure was particularly simple, involving only homogenization and sonication (Berdalat and Dorch 1991). More complex extraction procedures, however, may result in higher RNA and DNA yields than do simpler ones (McClark and Kaiser 1992). Simple additions to the technique which may result in increased yields include the addition of heparin or other proteases (e.g. Karsten and Wollenberger 1977), centrifugation steps and more complex washes (e.g. Clemeninos 1987) and detergents (sarcosyl, Calloside and Buckley 1991). For phytoplankton, Berdalat and Dorch (1991) found that the best recovery of TNA was obtained with Tri-Ca++ buffer, without the use of proteases, lysosymes, or detergents, although this has yet to be investigated in larval and juvenile fish.

RNA:DNA ratios are useful in detecting the occurrence of starvation in the ocean, on the basis of laboratory calibration (e.g. Buckley and Lough 1987; Canino et al. 1991). However, ratios have the potential either to produce spurious variation due to correlated errors or to size, or to absorb meaningful variation, causing the ratio to appear insensitive (Bergeron et al. 1991; Richaud et al. 1991; Mathers et al. 1994). Use of ANCOVA to determine homogeneity of slopes, and then generation of residuals from an overall regression, provides a relative, individual indices that is more controlled and specific to the technique and species. Comparisons between studies can be made concisely with the regression.

Acknowledgments

We gratefully acknowledge NSW Fisheries Port Stephens Research Centre, Salamander Bay, for the use of the hatchery facilities and for the support provided. This study was supported in part by a small ARC grant to I.M.S. We thank B. Talbot, P. Beevers and M. Lockhart for their technical assistance and support. We are also grateful to B. Sherwin, B. Houdin, P. J. Cincay, E. Berdalat and Bonnie Chan for their advice, and in particular to E. Cailardore and our referees.

References


Manuscript received 13 October 1995, revised and accepted 19 April 1996.