Freshwater Biology

Freshwater Biology (2011) **56,** 1736–1745 doi:10.1111/j.1365-2427.2011.02605.x

Eucalyptus leachate inhibits reproduction in a freshwater fish

JOHN R. MORRONGIELLO*,†, NICHOLAS R. BOND*,†, DAVID A. CROOK†,‡ AND BOB B. M. WONG*

*School of Biological Sciences, Monash University, Melbourne, Vic., Australia

†eWater Cooperative Research Centre, Canberra, ACT, Australia

‡Arthur Rylah Institute for Environmental Research, Department of Sustainability and Environment, Heidelberg, Vic., Australia

SUMMARY

1. Dissolved organic carbon (DOC) can induce lethal and sub-lethal effects in exposed biota via hypoxic blackwater events and the toxicity of leached compounds. Little is known of how DOC exposure affects fish reproduction despite the fact that its release can coincide with spawning-associated flow pulses.
2. River red gum (*Eucalyptus camaldulensis*) leaf leachate is a major source of DOC in Australian freshwaters and includes the toxic plant secondary metabolites polyphenols and tannins. High concentrations of leachate are released when leaves on floodplains or dry stream channels are inundated by water.
3. Southern pygmy perch (*Nannoperca australis*) from naturally high and naturally low *Eucalyptus* leachate environments in south-east Australia were exposed to elevated leachate levels to investigate the effects of DOC on reproduction and to explore whether response patterns were consistent with populations becoming locally adapted to historical leachate levels.
4. Fish exposed to leachate were half as likely to reach sexual maturity as control fish. Fish from a naturally high-exposure population tended to reach sexual maturity earlier than those from a naturally low-exposure population. Leachate exposure had no effect on either egg size or fecundity.
5. Our results suggest that leachate-exposed mothers did not reproduce because they were physiologically stressed or perceive the environment to be unsuitable, which raises the potential of plastic or adaptive responses to this stressor. The negative sub-lethal effects observed have important fitness implications for individuals, the viability of populations and the management of environmental flows and riparian zones.

Keywords: blackwater, environmental flow, flood pulse concept, floodplain, polyphenols

Introduction

The timing, magnitude and success of reproduction in many freshwater fish can be related to changes in temperature, day length, river discharge and the physiochemical environment (Humphries, King & Koehn, 1999; King, Tonkin & Mahoney, 2009; Hicks *et al*., 2010). Properties of the abiotic environment can also influence how a mother allocates resources to reproduction through a trade-off between egg size and egg number (reviewed by Bernardo, 1996). Given finite resources, theory predicts that mothers inhabiting poorer quality environments for offspring should produce fewer, larger eggs as the resulting (larger) offspring often have a fitness advantage through greater resource provisioning and stressor resistance

Correspondence: John R. Morrongiello, CSIRO Marine and Atmospheric Research, GPO Box 1538, Hobart, Tasmania 7001, Australia. E-mail: john.morrongiello@csiro.au

(Smith & Fretwell, 1974; McGinley, Temme & Geber, 1987). Indeed, the quality of environments experienced by mothers as juveniles (Taborsky, 2006) or adults (Johnston & Leggett, 2002) and the level of predictability and variability surrounding future offspring environments (Koops, Hutchings & Adams, 2003) have all been shown to influence the size and number of eggs produced.

Compounds found in dissolved organic carbon (DOC), such as polyphenols, tannins and humic substances, are common in many freshwater environments worldwide where they are leached from terrestrial (Suberkropp, Godshalk & Klug, 1976; Serrano, 1992; Chergui *et al.*, 1997) and aquatic plants (Gross, 2003). These compounds can be toxic to both aquatic invertebrates (Rey *et al.*, 2000; Canhoto & Laranjeira, 2007) and fish (Tremolieres, 1988; Steinberg *et al.*, 2006; McMaster & Bond, 2008) and are thus potentially important ecological and evolutionary drivers in freshwaters. Much of the DOC in Australian streams is derived from river red gums (*Eucalyptus camaldulensis* Dehnh.) (Francis & Sheldon, 2002), with over 90 chemical compounds identified in its leachate (Hillis, 1966; Cadahía *et al.*, 1997; Conde, Cadahia & GarciaVallejo, 1997; Farah *et al.*, 2002). *E. camaldulensis* shed leaves all year round, and high leaf biomass can build up on the floodplain and in dry stream channels during summer (Boulton & Lake, 1992; Reid *et al.*, 2008a). In unregulated river systems, rain or flow events in late winter and spring wet the deposited terrestrial organic material and cause DOC to leach into rivers and streams (Howitt *et al.*, 2007; Watkins, Quinn & Gawne, 2010). The resulting DOC pulse, including toxic polyphenols and tannins, often coincides with the onset of breeding in many south-east Australian native fish (Humphries *et al.*, 1999) and raises the potential for negative impacts on fish reproduction and subsequent recruitment.

The southern pygmy perch (*Nannoperca australis* Günther) is a small (<90 mm) fish found throughout south-east Australia, including Tasmania. It inhabits a range of freshwater environments and is naturally exposed to a gradient of *Eucalyptus* leachate concentrations (McMaster & Bond, 2008) related to heterogeneity of flow and tree cover (Howitt *et al.*, 2007; Watkins *et al.*, 2010). *N. australis* spawning is, in part, related to late winter and spring flow pulses (Tonkin, King & Mahoney, 2008; J.R. Morrongiello pers. obs.), so fish nearing reproductive maturity can be exposed to elevated levels of *Eucalyptus* leachate and DOC. Spatial variability in DOC exposure and significant genetic structuring (Hammer, 2001; Cook, Bunn & Hughes, 2007) also raises the possibility that *N. australis* populations may have become locally adapted to different levels of this environmental stressor (Kawecki & Ebert, 2004). High-exposure populations may then have evolved increased tolerance (Rasanen, Laurila & Merila, 2003) or modified their life histories (Lind, Persbo & Johansson, 2008) to ensure fitness is maintained. Patterns of larval *N. australis* tolerance to *Eucalyptus* leachate are not consistent with local adaptation, as larvae sourced from a low leachate environment had higher survival rates and faster growth rates than those from a high leachate environment when experimentally exposed to elevated leachate levels (J.R. Morrongiello unpubl. data). However, phenotypic patterns in male coloration (Morrongiello *et al.*, 2010) and egg size and fecundity (fecundity increases and egg size decreases with increasing stream ephemerality, J.R. Morrongiello unpubl. data) along environmental gradients suggest that trait variation in this species is adaptive. Indeed, it is possible that observed among-population variation in reproductive investment could in part be related to differences in *Eucalyptus* leachate exposure.

We explored experimentally the reproductive implications of chronic *Eucalyptus* leachate exposure using two *N. australis* populations from naturally high and naturally low leachate environments. We hypothesised that (i) mothers exposed to *Eucalyptus* leachate will be less likely to achieve sexual maturity, or will experience delayed maturity; (ii) mothers from higher leachate environments will be more likely to reach sexual maturity or mature earlier; and (iii) exposed mothers that do reproduce will produce fewer but larger eggs. These expectations are based on a number of presumptions. First, toxic compounds such as polyphenols and tannins in leachate are likely to elicit a physiological cost in exposed mothers by interfering with respiration (sensu Temmink *et al.*, 1989; Gehrke, Revell & Philbey, 1993). Second, adaptation or acclimation to elevated leachate levels will enhance a mother's tolerance to its toxic effects, meaning that reproduction is more likely to occur. Third, the larger larvae that are likely to originate from larger eggs will have greater stress resistance to leachate.

Methods

Experimental design

Twenty-four outdoor mesocosms were prepared 2 months prior to the start of the experiment. Each mesocosm (bathtubs, average dimensions: 138.8 × 60.6 × 31.4 cm, 264.1 L) was half-buried in the ground and fitted with a standpipe to maintain water level, provided with a sand substrate and two potted aquatic plants (*Myriophyllum* sp. and *Vallisneria* sp.) and inoculated with zooplankton (*Daphnia* spp., ostracod spp. and copepod spp.) collected from a nearby lake. During the 2 months, mesocosms were aerially colonised by aquatic insects. At the start of the experiment, shade cloth was placed over each mesocosm to prevent bird access and provide additional cover for fish.

In July 2009, 312 immature *N. australis* were collected from Castle Creek (36°51′58″S, 145°35′9″E) and Broken River (36°58′33″S, 146°6′28″E) using fyke nets and stocked at a density of 13 fish per mesocosm. At the time of spawning, Castle Creek fish are generally exposed to more elevated levels of *Eucalyptus* leachate compared to those from Broken River (samples taken from multiple reaches in each stream: 22.57–22.78 mg L^{-1} polyphenol over ~22 km versus 0.53–0.78 mg L^{-1} over ~9 km; J.R. Morrongiello, unpubl. data), and this difference is likely to persist to some degree throughout the spawning season (elevated DOC in late summer and consistent water spectral properties over 1 month in Castle Creek: McMaster & Bond, 2008; Morrongiello *et al.*, 2010). Spatial differences in leachate exposure are probably attributed to a more intact riparian zone and ephemeral hydrology at Castle Creek that facilitates the build-up of organic material and subsequent leaching of DOC. The fish were allowed to acclimate for 2 days, after which an *E. camaldulensis* leachate treatment was randomly applied to half the mesocosms and the remaining left as controls with untreated water. Thus, there were six water treatment replicates within each population. Fish in each mesocosm were fed 10 g of frozen bloodworms three times a week to supplement the existing macroinvertebrate diet.

Treatment application

Field measurements and laboratory experiments found that a polyphenol concentration of 20 mg L^{-1} represented an elevated, yet ecologically relevant, level of *Eucalyptus* leachate that caused significant lethal and sub-lethal effects in larval *N. australis* (J.R. Morrongiello unpubl. data). We therefore adopted 20 mg L^{-1} polyphenol as our treatment level in this experiment. Terrestrially aged *E. camaldulensis* leaves were collected from the Castle Creek riparian zone and submerged in blackened tubs for 20 days to leach DOC (O'Connell *et al.*, 2000). Polyphenol concentration of this master solution was colorimetrically measured as gallic acid equivalents using a modification of the Folin–Ciocalteau method (Forrest & Bendall, 1969). Initially, 0.5 mL of 40 μm filtered leachate was mixed with 0.5 mL of Folin–Ciocalteau reagent (Sigma-Aldrich, Castle Hill, NSW, Australia) and buffered with 5 mL of 1 M Na_2CO_3 in a 25-mL test tube. These tubes were agitated and allowed to react for 1 h. Subsequently, sample absorbance was measured at 765 nm on a Cary 50 UV–Vis spectrophotometer (Varian Inc., Walnut Creek, CA, U.S.A.) and compared to gallic acid standards. The treatment was not reapplied during the experiment to reflect the natural processes of leachate degradation (Howitt *et al.*, 2008) and dilution through time following initial DOC release from inundated *E. camaldulensis* leaves.

Reproductive measurement

The experiment was completed after 3 months when on 15 October 2009 remaining fish (recovery rate 76%) were caught. Fish were killed by administering an overdose of clove oil, and total length was measured to the nearest millimeter. Mature eggs were expressed from females by gently squeezing their abdomen and allowed to water harden for approximately 10 min. These eggs were photographed with a Nikon D80 camera (Nikon Inc., Tokyo, Japan) fitted with a macrolens. Fish were then preserved in 95% ethanol.

The cross-sectional areas of individual eggs from each fish were measured from digital photographs using IMAGE J version 1.38x (NIH, U.S.A., rsbweb.nih.gov/ij) and converted into diameters (mm). Larger eggs have bigger yolks (J.R. Morrongiello unpubl. data) and therefore are assumed to represent a greater level of maternal resource investment. Egg size is the average egg diameter within a female. Fecundity was calculated by summing the number of mature eggs in both ovaries with eggs that had been expressed by the female for egg size measurements. A female's maturity status (ripe, unripe) was ascertained by a visual inspection of the gonad (Humphries, 1995) and used to calculate the proportion of ripe females in each mesocosm.

Reproductive data analysis

The effect of treatment and population on the proportion of ripe females was analysed using logistic regression weighted by sample size. The model was fitted with a quasi-binomial distribution to account for overdispersion, and the significance of terms was tested using F statistics (Logan, 2010).

Variation in egg size and fecundity was explored using linear mixed effect models to account for the hierarchical nature of the data (Zuur *et al.*, 2009). For each trait model, treatment (control, leachate), population (Castle, Broken) and length were fitted as fixed effects, mesocosm was treated as a grouping random effect, and the 24 length by trait slopes were allowed to vary randomly among mesocosms. Random effect structures were explored using restricted maximum likelihood estimates of error (REML) fitted to the full interactive fixed effect model (Zuur *et al.*, 2009) and their relative support assessed using Akakie's Information Criterion corrected for small sample sizes (AICc; Burnham & Anderson, 2002). For both egg size and fecundity, a random effect structure including just the mesocosm random intercept and fixed slopes for length performed best. Sixteen models of increasing fixed effect complexity were fitted to each trait using maximum likelihood estimates of error and their performance compared using AICc. The optimal model was reanalysed using REML to produce unbiased parameter estimates (Zuur *et al.*, 2009). Variation in length among populations and treatments was explored using the same procedure, with four models of increasing fixed effect complexity fitted to the data. Egg size, fecundity and length were all natural log transformed to satisfy model assumptions, and analysis was performed using the nlme package (3.1-96) in R 2.12.0 (R Development Core Team, 2010).

Physiochemical parameters

Dissolved oxygen (mg L^{-1}) was measured at approximately 10 am on six occasions during the experiment (days: 0, 7, 28, 49, 70 and 92) using a Hach LDO probe (Hach Company, Loveland, CO, U.S.A.) and analysed using a mixed effects linear model. Treatment and days were fitted as fixed effects and mesocosm as a random grouping effect to account for the repeated measures nature of the data. Model selection and parameter estimation followed the procedure mentioned earlier. Temporal trends across replicates (days) were explored using polynomial contrasts (Quinn & Keough, 2002).

Results

A total of 74 female *N. australis* reached sexual maturity in the mesocosms. We obtained estimates of egg size and fecundity for 56 and 74 of these, respectively. One mesocosm (Broken River leachate) developed a leak during the experiment and was omitted from further analysis.

There was no significant interaction between population and treatment ($F_{1,19} = 0.34$, $P = 0.57$) in the proportion ripe model so this term was dropped from the analysis. At the time of sampling, leachate-exposed fish were 55% less likely to be sexually mature than control fish (odds ratio on logit scale: 0.162, $F_{1,21} = 14.26$, $P = 0.001$), and Castle Creek fish were approximately 7% more likely to be sexually mature than Broken River fish (odds ratio: 2.558, $F_{1,21} = 4.09$, $P = 0.057$) (Fig. 1).

The model best explaining variation in egg size included population and length (Table 1). Broken River fish had larger eggs than Castle Creek fish (Fig. 2), and bigger fish produced larger eggs (Fig. 3a). Variation in fecundity was just related to length (Table 1), with bigger fish producing more eggs (Fig. 3b). Broken River fish were bigger than those from Castle Creek (table 1). Treatment did not affect any of these traits.

The model best explaining temporal variation in dissolved oxygen included the additive effects of treatment ($F_{1,21} = 43.63$, $P < 0.001$) and days ($F_{5,110} = 4.81$, $P < 0.001$). Control mesocosms had higher dissolved oxygen than leachate mesocosms, and the magnitude of this difference changed through time (cubic trend $P < 0.012$, Fig. 4). Leachate mesocosms experienced an initial decline in dissolved oxygen from approximately 7 mg L^{-1} to 4 mg L^{-1} after which concentrations stabilised, whilst dissolved oxygen remained relatively constant through time in control mesocosms.

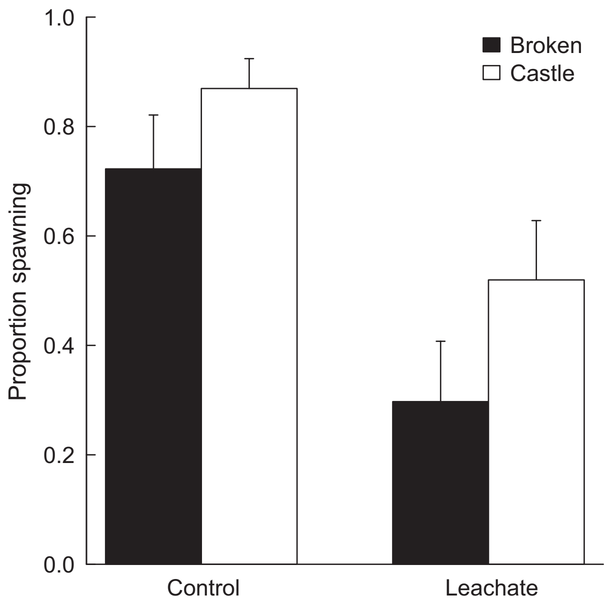

Fig. 1 Mean (±SE) proportion of female *N. australis* from Broken River and Castle Creek spawning when exposed to control and leachate (20 mg L^{-1} polyphenol) water.

Discussion

Female *N. australis* that were chronically exposed to *Eucalyptus* leachate were half as likely to reach sexual maturity compared to control fish. This substantial negative effect may be attributed to a number of mechanisms. Leaf leachates, in particular polyphenols and tannins, are known to be toxic to a variety of fish species, and our result may reflect the physiological stress of exposed mothers. For example, when carp (Temmink *et al*., 1989) and golden perch (Gehrke *et al*., 1993) were exposed to bark and leaf extracts, their gills became damaged and this likely interfered with respiration. Likewise, acute and chronic *Eucalyptus* leachate exposure affected the behaviour and survival of a range of Australian native fish, including *N. australis* (Gehrke *et al*., 1993; McMaster & Bond, 2008). Exposed females presumably would have to expend considerable energy to resist the effects of leachate, thus reducing the resources available for allocation to reproduction. Leachate exposure had no effect on final maternal size, which suggests that the difference in sexual maturity between treatments was not attributable to some individuals failing to reach a minimum size for maturity. This proposition is further supported by the tendency of Castle Creek females to mature more readily despite being significantly smaller than Broken River females.

Alternatively, females exposed to *Eucalyptus* leachate may have perceived the environment to be of poor quality, where offspring survival was likely to be low, and thus did not invest as much resources into reproduction. Such a phenotypically plastic response may enable mothers to conserve resources and potentially spawn at a later date when conditions are more favourable. In a similar vein, the absence of cues related to environmental conditions experienced by larvae has been implicated in the failure of Australian grayling and golden perch to spawn in a given season (Lake, 1967; O'Connor & Mahoney, 2004) and the postponement of spawning in others (reviewed in Humphries *et al*., 1999). If spawning is delayed rather than prevented by leachate exposure, larvae hatching later may still experience fitness costs as they are exposed to greater competition from more developed conspecifics (Post, 2003), mismatch with food availability because of temporal changes in stream fauna (Boulton & Lake, 1992) or miss the opportunity to disperse on flow pulses to more favourable habitat (Perry & Bond, 2009). Regardless of the causal mechanism, our results suggest that elevated DOC levels and blackwater events associated with floodplain inundation or channel rewetting (Howitt *et al*., 2007) can negatively impact upon the

Table 1 Parameter estimates and test statistics from the most parsimonious linear mixed effect models testing for differences in egg size, fecundity and length

Parameter	Estimate (SE)	df	*F* statistics	*P* value
Log egg size				
Castle versus Broken populations	−0.026 (0.014)	1.19	10.279	0.004
Log length	0.138 (0.034)	1.34	16.814	<0.001
Log fecundity				
Log length	2.892 (0.415)	1.54	48.549	<0.001
Log length				
Castle versus Broken populations	−0.100 (0.034)	1.21	8.550	0.008

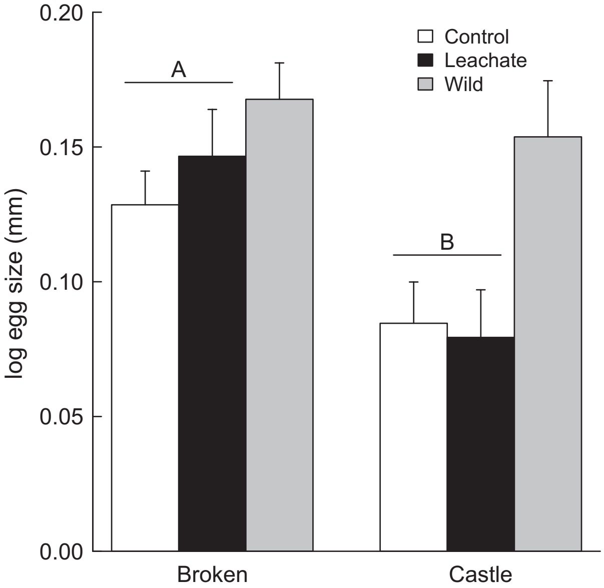

Fig. 2 Mean (±SE) egg size produced by *N. australis* females from Broken River and Castle Creek when exposed to control and leachate (20 mg L^{-1} polyphenol) water. Letters indicate significant differences. 'Wild' egg sizes refer to field observations for these populations (J.R. Morrongiello unpubl. data).

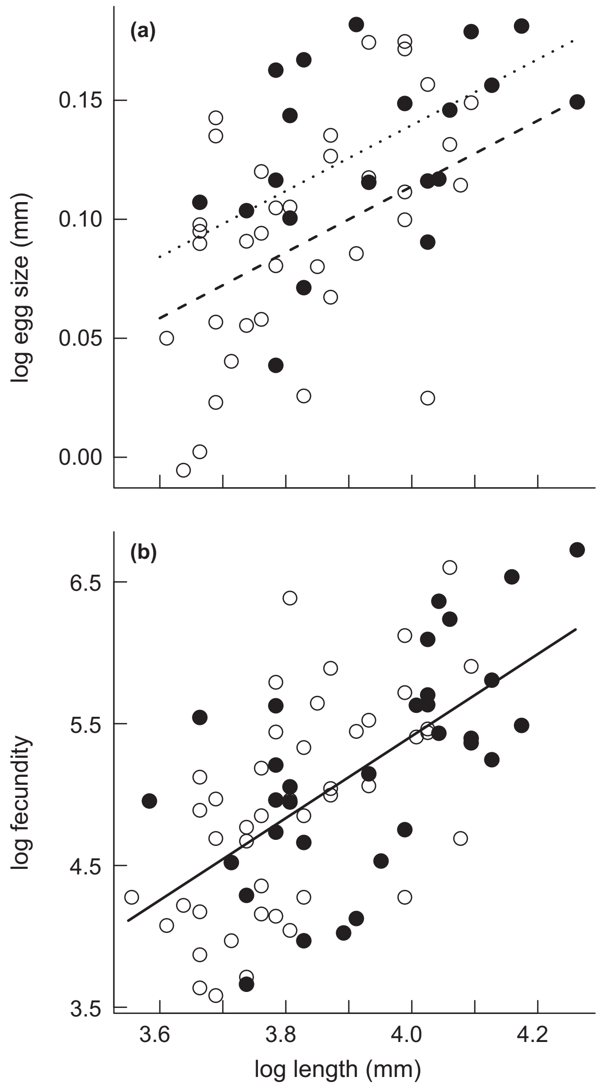

Fig. 3 Relationship to length of (a) egg size and (b) fecundity in *N. australis* from Broken River (filled circles) and Castle Creek (open circles), pooled across treatments. The lines represent predicted relationships from linear mixed effect models (…, Broken River egg size; —, Castle Creek egg size; —, pooled fecundity). Each point represents an individual fish.

breeding and recruitment of fish that spawn on increased discharge events (Tonkin *et al.*, 2008; King *et al.*, 2009).

Previous studies of the interactions between DOC and fish have often identified low dissolved oxygen levels as an important codriver of observed impacts. The rapid consumption of DOC by microbes can result in hypoxic conditions leading to large-scale fish deaths (Townsend, Boland & Wrigley, 1992; Townsend & Edwards, 2003). Likewise, Gehrke *et al.* (1993) and McMaster & Bond (2008) identified interactive effects between leachate exposure and dissolved oxygen in laboratory experiments. However, the results of this study and those reported by Fisher, Wong & Rosenthal (2006) and Rey *et al.* (2000) indicate that DOC compounds, such as the polyphenols and tannins in *Eucalyptus* leachate, can negatively affect fish without involving hypoxia.

The tendency, albeit weak, for a greater proportion of Castle Creek fish to mature when housed under the same conditions as Broken River fish could suggest that historical *Eucalyptus* leachate exposure patterns may influence *N. australis* reproduction and may indicate some local adaptation. As Castle Creek fish are naturally exposed to higher leachate levels because of a more intact riparian zone and ephemeral hydrology (J.R. Morrongiello unpubl. data), it follows that these fish will be more likely to spawn when confronted with elevated DOC levels (Kawecki & Ebert, 2004). Conversely, the observed population differences may be owing to Broken River females delaying maturation until conditions improve or to Castle Creek fish naturally maturing earlier than Broken River fish and thus biasing results. However, field observations suggest that both populations actually commence

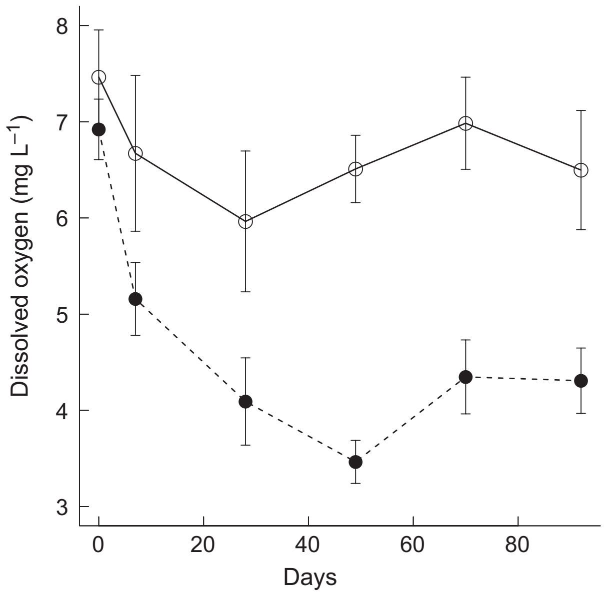

Fig. 4 Temporal changes (mean ± SE) in dissolved oxygen concentrations from control mesocosms (open circles) and mesocosms with added leachate (filled circles).

spawning at similar times (J.R. Morrongiello pers. obs.). Moreover, Broken River larvae had greater resistance to *Eucalyptus* leachate than those from Castle Creek (J.R. Morrongiello unpubl. data), which, taken with the small effect size observed here (7%), tempers any suggestion of local adaptation.

Contrary to our predictions, exposure to *Eucalyptus* leachate had no effect on either egg size or fecundity, even though previous work had shown that larger *N. australis* larvae were less likely to die than smaller larvae when exposed to elevated levels of leachate (J.R. Morrongiello unpubl. data). Larger eggs have greater yolk volumes (J.R. Morrongiello unpubl. data), and it is likely that larger or fitter offspring hatch from these eggs as occurs in other fishes (e.g. Einum & Fleming, 2000). It is possible that leachate-exposed mothers were too physiologically stressed to allocate additional resources to offspring or that allocation 'decisions' were already made as juveniles and thus not necessarily affected by the adult experience of this experiment (Taborsky, 2006). Whilst Broken River females produced eggs of similar size to those of wild Broken River females, Castle Creek eggs in this experiment were substantially smaller than previously recorded (Fig. 2). A similar pattern of smaller eggs being produced in the mesocosms was observed in a 2008 pilot study (J.R. Morrongiello, unpublished data) and suggests that egg size is a phenotypically plastic trait within and among populations. The lack of a consistent treatment effect in this experiment indicates that observed correlative patterns among egg size and fecundity along hydrological gradients (J.R. Morrongiello unpubl. data) are not related to differences in natural leachate exposure. Positive relationships between female body size and fecundity or egg size are common in fishes (e.g. Johnston & Leggett, 2002) and likely reflect allometric constraints (Wootton, 1998) or maternal phenotypic influences, such as resources available for reproduction or oviposition sites (reviewed in Marshall *et al.*, 2010).

The effects of DOC on aquatic biota are complex and contextually dependent. The chemical composition and toxicity of leaf extracts substantially change through time and are reliant on the degree of leaf ageing prior to wetting (Tremolieres, 1988; Baldwin, 1999; O'Connell *et al.*, 2000). Whilst exposure to freshly released DOC induces acute toxic effects in fish (Temmink *et al.*, 1989; Gehrke *et al.*, 1993), longer-term persistence of derived compounds in the freshwater environment is more likely to cause chronic effects, such as those observed here. Indeed, the timing, duration and magnitude of DOC exposure will determine whether biota display lethal or sub-lethal responses. Elevated DOC levels can also be beneficial to fish reproduction and larval survival. Huff, Grad & Williamson (2004) found that the eggs of yellow perch are killed by ultraviolet radiation, but elevated DOC levels blocked much of this and allowed fish to spawn successfully at shallower depths where water temperatures optimised larval development. Likewise, a boost in secondary productivity associated with DOC leaching and the submersion of terrestrial organic matter (O'Connell *et al.*, 2000; Reid *et al.*, 2008b) will provide an important food source for larval fish. The release of DOC is related to hydrology and organic matter availability (Francis & Sheldon, 2002; Howitt *et al.*, 2007), and this has obvious implications for the management of riparian zones and flow in regulated systems (Reid *et al.*, 2008a; Watkins *et al.*, 2010).

Our findings provide a new perspective on the impacts DOC can have on aquatic biota. We have shown that DOC exposure can interfere with fish reproduction, which, in turn, has implications for the fitness of individuals and the viability of populations. Together with results from previous studies, this reinforces the need to understand better the significant and pervasive negative impacts DOC can have on ecological and evolutionary outcomes in aquatic environments.

Acknowledgments

We thank M.R. and T. Morrongiello for mesocosm construction and fish collection, H., A. and M.C. Morrongiello for data collection and J. Read for laboratory space. H. Morrongiello, R. Jones and anonymous reviewers provided valuable comments that improved this manuscript. This research was financially supported by eWater CRC, the Australian Research Council and Monash University. J.R. Morrongiello was supported by an Australian Postgraduate Award (I) scholarship during this research. Collection and handling of fish was conducted under Monash University School of Biological Sciences Ethics Committee approval (SCI 202007/08) and Fisheries Victoria permit RP 882.

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(Manuscript accepted 11 March 2011)