

Trematode infection causes malformations and population effects in a declining New Zealand fish

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Summary

1. Animal malformations engender wide public and scientific concern because of associated environmental health risks. This is highlighted by increased incidence of limb malformations in amphibians associated with trematode infections and disturbance. Malformations may signal new emerging disease threats, but whether the phenomenon is broadly applicable across taxa, or has population-scale impacts, is unknown.
2. Malformations are widely reported in fish and, until now, have been attributed mainly to contaminants. We tested whether the trematode *Telogaster opisthorchis* caused severe malformations, leading to population effects, in *Galaxias anomalus*, a threatened New Zealand freshwater fish.
3. Experimental infection of larval fish caused increasing spinal malformation and mortality with infection intensity that closely matched field patterns. Field malformation frequency peaked in January (65%), before declining sharply in February (25%) and remaining low thereafter.
4. The peak occurred during a ‘critical window’ of larval development, with the decline coincident with a population crash, indicating that malformation was causing mortality in the field.
5. The occurrence of such critical developmental windows may explain why this mechanism of population impact has been overlooked. With global environmental stressors predicted to enhance trematode infections, our results show that parasite-induced malformation, and its population-scale impacts, could be more widespread than previously considered.

Key-words: disease, fish, infection, malformation, mortality, population, trematode

Introduction

Global biodiversity loss has been attributed to a wide range of factors among which emerging diseases are an increasing concern (Daszak, Cunningham & Hyatt 2000; Tompkins, White & Boots 2003; de Castro & Bolker 2005). The identification of causal links between disease and host population changes is an important conservation goal but is complicated by multiple stressors, and the difficulty of sampling infected individuals for diseases that cause rapid mortality (Lafferty & Holt 2003; McKenzie & Townsend 2007; Jacobson *et al.* 2008; Kelly *et al.* 2009a). Sub-lethal effects can, however, be useful bio-indicators of disease risk (Johnson *et al.* 1999; Delgado-Acevedo & Restrepo 2008). For instance, animal developmental malformations have attracted wide public and scientific attention because they often act as indicators of environmental perturbation and contaminants that could pose risks to both human and wildlife health (Lemly 1997; Blaustein & Johnson 2003; Hu *et al.* 2009).

Apparent increases in limb malformations in amphibians highlight concerns that environmental stressors could be partly responsible for associated population declines (Johnson *et al.* 1999; Blaustein & Johnson 2003; Rohr *et al.* 2008). Strong experimental evidence has linked these limb malformations to infections by parasitic trematodes (Sessions & Ruth 1990; Johnson *et al.* 1999; Blaustein & Johnson 2003; but see Skelly *et al.* 2007). Aquatic snails release infective cercariae of the trematode *Ribeiroia ondatrae* that encyst as metacercariae in the limb buds of juvenile amphibians. Infection impairs limb development resulting in missing and extra limbs or digits, with the frequency of malformation positively correlated with infection intensity (Johnson *et al.* 1999; Blaustein & Johnson 2003). As trematode transmission depends on the availability of aquatic snails and the temperature-sensitive production of infective cercariae, global climate and land-use change are expected to increase impacts of such trematode-induced malformations on aquatic communities (Kiesecker 2002; Johnson & Chase 2004; Poulin 2006; McKenzie & Townsend 2007; Rohr *et al.* 2008).

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Amphibian limb abnormalities have undoubtedly raised awareness of disease factors that threaten animal populations. However, we have limited understanding of the general importance of the phenomenon in other taxa. Furthermore, although trematode-induced malformations may indicate a new emerging disease threat to wildlife, whether trematode-induced malformations have population scale effects is unknown (Blaustein & Johnson 2003; Johnson & Sutherland 2003). Here, we address both of these issues in an investigation of trematode-induced malformation, and its implications for population recruitment, in a freshwater galaxiid fish.

Fish are the most diverse of the vertebrate groups. Many populations are seriously threatened, ostensibly due to climate change, habitat modification and the introduction of non-indigenous species (Duncan & Lockwood 2001; Olden, Hogan & Vander Zanden 2007). Fish are thus important indicators of aquatic ecosystem stress; indeed, developmental malformations have been widely reported (e.g. Lemly 1997; Hu *et al.* 2009). Contaminants are considered the primary cause of such malformations in natural freshwater systems (Maier & Knight 1994; Hu *et al.* 2009), but there is increasing evidence that trematode infections may have similar impacts (e.g. Cunningham *et al.* 2005).

Galaxiids are the most speciose of the temperate freshwater fish taxa in the southern hemisphere, exhibiting their greatest richness and endemism in New Zealand (McDowall 2006). They are also one of the most at risk groups of freshwater fishes; a large number of non-migratory species are threatened by habitat modification and pollution, and the introduction of non-indigenous fish (Duncan & Lockwood 2001; Townsend 2003; McDowall 2006). During stream surveys conducted in the South Island of New Zealand in November 2008, we observed severe spinal and fin malformations in adult *Galaxias anomalus* Stokell, a threatened freshwater fish native to New Zealand (McDowall 2006) (Fig. 1); many fish were heavily infected by metacercarial cysts of the trematode *Telogaster opisthorchis* Macfarlane, 1945. As with many trematode infections of amphibians, *Galaxias* species are second intermediate hosts for metacercariae that encyst in the fish musculature after their release from aquatic snails (Kelly *et al.* 2009b). Completion of the life cycle requires transmission to a definitive host (freshwater eel or bird) by predation on small infected fish. Although there are few formal accounts of malformations in galaxiids, Allibone (2000) reported head, jaw and fin malformations in two different *Galaxias* species in New Zealand, while in archive samples malformations of *Galaxias* are widespread in New Zealand's South Island (D. Kelly, unpublished data).

In this study, we use field surveys and controlled laboratory experiments with post-larval fish to test the hypothesis that trematode metacercariae cause severe spinal and fin malformations in *G. anomalus*. We also investigate the influence of trematode-associated malformation and mortality on the temporal dynamics of a new fish cohort. Early life stage survival is considered a key determinant of fish population



Fig. 1. Spinal malformations in adult *Galaxias anomalus* with natural infections of *Telogaster opisthorchis* (the bottom fish is normal).

recruitment (e.g. Cushing 1996), and several studies have shown parasite impacts on juvenile stages (e.g. Lemly & Esch 1984; Collyer & Stockwell 2004; Jacobson *et al.* 2008). Thus, if trematode-induced abnormalities impact mainly larval or early juvenile fish stages, then they could have strong effects on the dynamics of fish populations.

Materials and methods

FIELD SURVEY

Our survey was conducted in an unnamed spring-fed stream of the Upper Taieri River in New Zealand's South Island (45°06'45''S, 170°15'18''E). Monthly sampling (December 2008 to April 2009) was used to estimate the size of the *G. anomalus* population and the relationship between the frequency of abnormalities and the intensity of infection. The sampling period encompassed larval to juvenile metamorphosis when fish were *c.* 2–3 weeks old (see Benzie 1968). Fish were quantitatively surveyed from two stream reaches (avg. length × width × depth; 10 × 2.0 × 0.2 m and 14 × 1.5 × 0.15 m respectively), each separated by 25 m stream length, using three pass removal sampling (Kelly & Dick 2005). Fish were sampled using a sweep net (1 m width, 2 × 2 mm mesh) rather than conventional electro-fishing. Each reach was divided into small sections (2 m length) that were successively sampled in an upstream direction. In each section, an operator disturbed the area so that fish were herded downstream to the net. Fish were held in stream-side bins (50 L), with the number of adults and juveniles collected per pass recorded. It was not possible to accurately estimate juvenile density in December because the fish inhabited dense macrophytes and were highly vulnerable to mortality during collection. We therefore collected a sub-sample of

juveniles in December by sweeping the net several times through the macrophytes. On each sampling occasion between January and April, a random sub-sample of juvenile fish was taken and fixed in 10% buffered formalin. A sub-sample of adults was taken only in December 2008; in subsequent months (January–April), adult malformations were assessed visually on site (see below), after which fish were returned alive to the stream. Fish population size was estimated using Zippin's maximum likelihood method and converted to density based on estimated reach area (Cowx 1983).

To assess spinal malformations and infection intensity of *Telogaster* metacercariae, fish were dehydrated by immersion in increasingly concentrated ethanol solutions (70%, 95%, 98%, 100%, 100% – each for 2 h), followed by tissue clearing in clove oil. Body length, the extent of spinal malformation, the presence and types of fin abnormality, and the total number of *Telogaster* metacercariae anywhere in the fish were recorded for each individual. If the spine was straight,

without signs of curvature (see Fig. 2a,b), then a fish was scored zero for spinal malformation; for fish with abnormal spines, the total number of scoliotic (lateral) and lordotic (dorso-ventral) deviation points along the spine were counted (Fig. 2c–h). Fin malformations, which were observed only for paired pelvic or pectoral fins, were categorized as follows:

1. Extra fins (in addition to the normal two) in the paired pelvic fin region (Fig. 2i,j);
2. Missing fins (one instead of two);
3. Size asymmetry; one fin $< 2/3$ length of the other;
4. Fins asymmetrically opposed.

For adult fish that were assessed visually on-site, we recorded only the frequency of fish with spinal malformations. Also, we did not assess paired fin abnormalities in adults between January and April

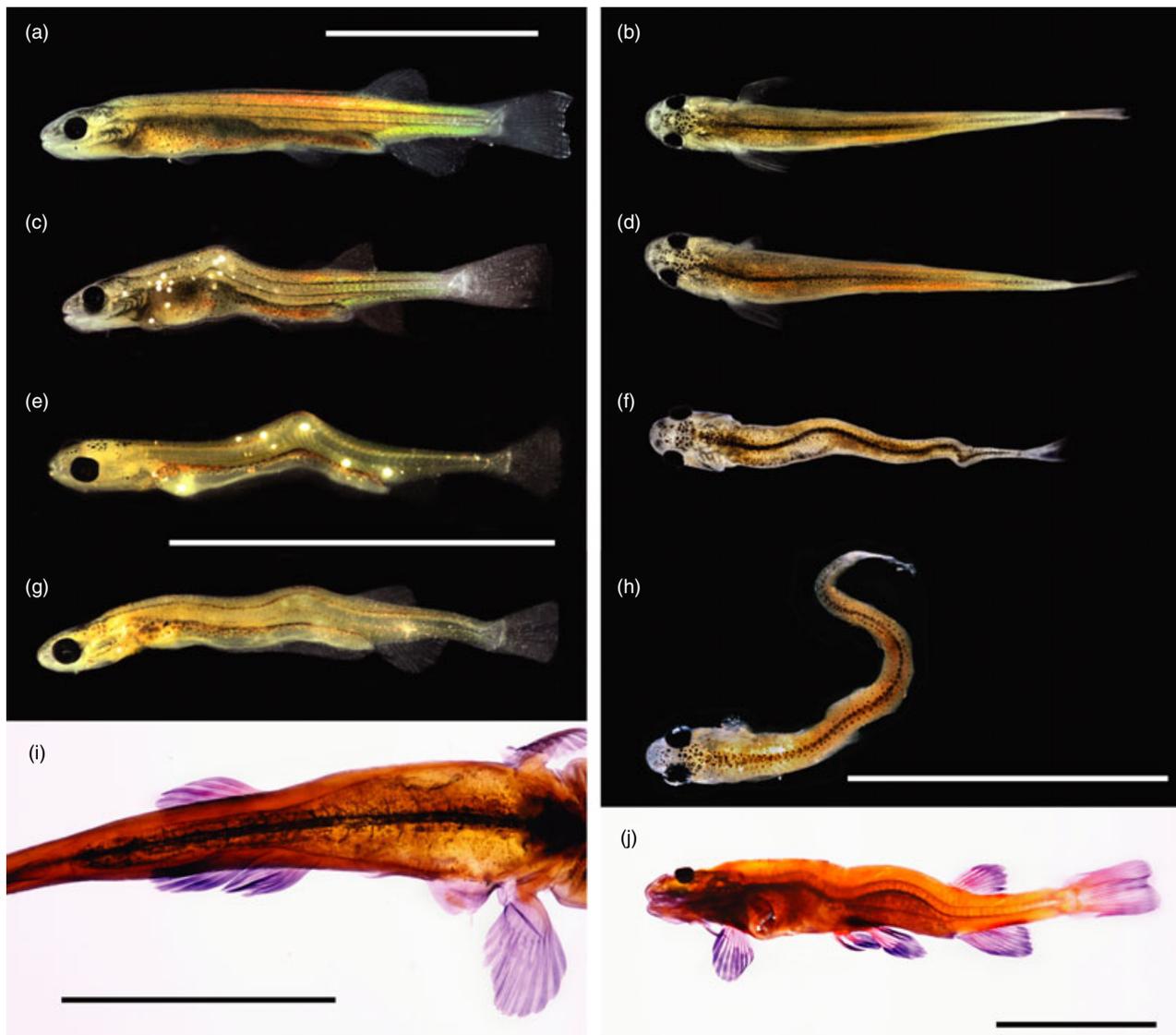


Fig. 2. Normal and malformed late larval (2–3 weeks post-hatch) and juvenile (3–6 weeks post-hatch) *Galaxias anomalus* from the wild. Normal dorso-ventral (a) and lateral (b) spinal development. Dorso-ventral malformations of one (c and e) and two (g) spinal deviations (*Telogaster opisthorchis* metacercariae) can be clearly observed as white circles in c and e. Lateral malformations of one (d), five (f) and four (h) spinal deviations. Fin malformations of one (i) or three (j) additional pelvic fins. Scale bars represent 10 mm; for spinal malformation images, the top scale bar refers to fish a–d, and f, the middle bar fish e–g, and the lower bar fish h.

because of the difficulty of inspecting live fish and our concern to return fish unharmed to the stream.

To assess whether trematode infections accumulated, and juvenile fish size (a surrogate of age) increased over the sampling period, single-factor ANOVAS examined the effect of sampling month on both fish body length and infection intensity (number of *T. opisthorchis* cysts). To investigate whether spinal malformations in juvenile fish were related to infection intensity and whether the pattern changed as fish grew, a general linear model assessed both the additive and interactive effects of sampling month (a surrogate of fish size; see Results) and infection intensity on the number of spinal deviations per fish. Predictor and response variables were appropriately transformed [arcsine square root or $\log_{10}(x + 1)$] prior to analysis. To further investigate interactive effects between sampling period and *T. opisthorchis* infection, separate regression analyses examined the number of deviations per fish vs. infection intensity for each sampling month, controlling for potential confounding effects of fish size by analysing the residuals from regressions of the number of deviations on fish length.

LABORATORY INFECTIONS OF JUVENILE GALAXIAS ANOMALUS

To provide a source of trematode infection *Potamopyrgus antipodarum* (Gray, 1853), the snail intermediate host of *T. opisthorchis*, were collected from our study site in December 2008 and housed in laboratory aquaria at 12 °C. Infected snails were identified by placing individuals in 12-well plastic trays (12 per well), incubating them at 16 °C under intense light, and screening for cercariae after 24 h. Snails from positive wells were separated into individual wells and screened for a further 24 h, after which infected snails were placed in separate aquaria (see below). Snails not shedding cercariae were repeatedly screened and considered uninfected if no cercariae were shed after five attempts.

The experiment was conducted during the austral summer, coincident with the onset of the field survey (December 2008–January 2009). Owing to a high prevalence of *T. opisthorchis* infection in a sub-sample of juvenile *G. anomalus* collected from our study site in November (42%, $n = 14$), individuals for the infection experiment were collected from a site of low prevalence ($n = 119$; 3.3%) 8 km away from the field study site (45°08'50"S, 170°17'35"E). Fish were maintained in large holding aquaria for 1 week and fed on fine commercial pellet food prior to experimental infections, at which stage they were *c.* 3–4 weeks old. Four fish were randomly allocated to each of 32.2 L experimental aquaria and allowed to acclimatize for 2 days. Snails were added to aquaria in a 2 × 2 factorial design [low (two snails) vs. high (five snails) abundance; snails infected vs. uninfected], giving four experimental treatments with eight replicates each. Fish were fed fine pellets (twice daily); snails were fed algal pellets (every 3 days). Snails were allowed to shed cercariae naturally, with the experiment running for 32 days during which each replicate was monitored twice daily. At the end of the experiment all fish (including those that died prematurely) were assessed for both abnormalities and the number of encysted metacercariae, using the methods described above.

Mean infection intensity, the severity of spinal malformation, the frequency of fin abnormalities and mean per cent fish survival were compared between treatments with respect to snail abundance and infection status in two-factor ANOVAS. All data was either $\log_{10}(x + 1)$ or arcsine square root transformed prior to analysis as appropriate.

Results

FIELD SURVEY

Juvenile *G. anomalus* densities declined over the sampling period with the steepest declines occurring between January and February, and between March and April (Fig. 3a). Spinal malformation frequency increased from December to the highest level in January (65%) before declining dramatically between January and February, after which the level remained consistently low (Fig. 3b). Fin malformations in juvenile fish occurred at lower frequency than spinal malformations and, although absent in December, remained consistent from January to April (see Table 1). No juveniles had missing fins. Mean juvenile fish length increased over the sampling period ($F_{4,326} = 243.6$, $P < 0.0001$; Fig. 3c), accompanied by an increase in the mean number of *T. opisthorchis* metacercariae per fish ($F_{4,326} = 272.7$, $P < 0.0001$; Fig. 3c). Most juvenile fish were infected in December (85%), with all becoming infected from January onwards (Table 1).

Severity of spinal malformation increased significantly with infection intensity ($F_{1,321} = 18.9$, estimate 0.28, $P < 0.0001$), although the strength of the relationship varied between sampling months (infection intensity × sampling month interaction; $F_{1,321} = 3.81$, $P = 0.005$). In individual regressions for each month, the severity of spinal malformation was positively related to infection intensity in December and January ($r = 0.64$, d.f._{1,53}, and $r = 0.47$, d.f._{1,114}, respectively, both $P < 0.0001$; Fig. 4a,b), but not in February, March or April (respectively, $r = 0.09$, d.f._{1,67}, $r = 0.03$, d.f._{1,69}; and $r = 0.36$, d.f._{1,21}; all NS, Fig. 4c–e).

Adult *G. anomalus* densities declined between December and January, after which they remained consistently low (Fig. 3a). All adult fish were infected with trematode metacercariae but had much lower frequencies of spinal malformations than juvenile fish, with no apparent trend over time (Fig. 3b). Fin malformations in adults, which could be assessed only in December, occurred at similar frequency to spinal malformations, and included such malformations as size asymmetry (18.1%) and extra fins (up to six pelvic fins in 9% of fish) or missing fins (4.5%).

LABORATORY INFECTIONS OF JUVENILE GALAXIAS ANOMALUS

Both snail infection status ($F_{1,28} = 800.1$, $P < 0.0001$), and its interaction with snail abundance ($F_{1,28} = 6.23$, $P < 0.05$), were significantly related to the intensity of *T. opisthorchis* infection established in fish (Fig. 5a). Fish exposed to infected snails became infected, whereas those exposed to uninfected snails remained uninfected, and exposure to a higher abundance of infected snails resulted in significantly more *T. opisthorchis* metacercariae per fish (Fisher's protected least significant difference *post hoc* test, $P < 0.01$). The level of infection established under both treatments fell well within the observed natural range (Fig. 3c).

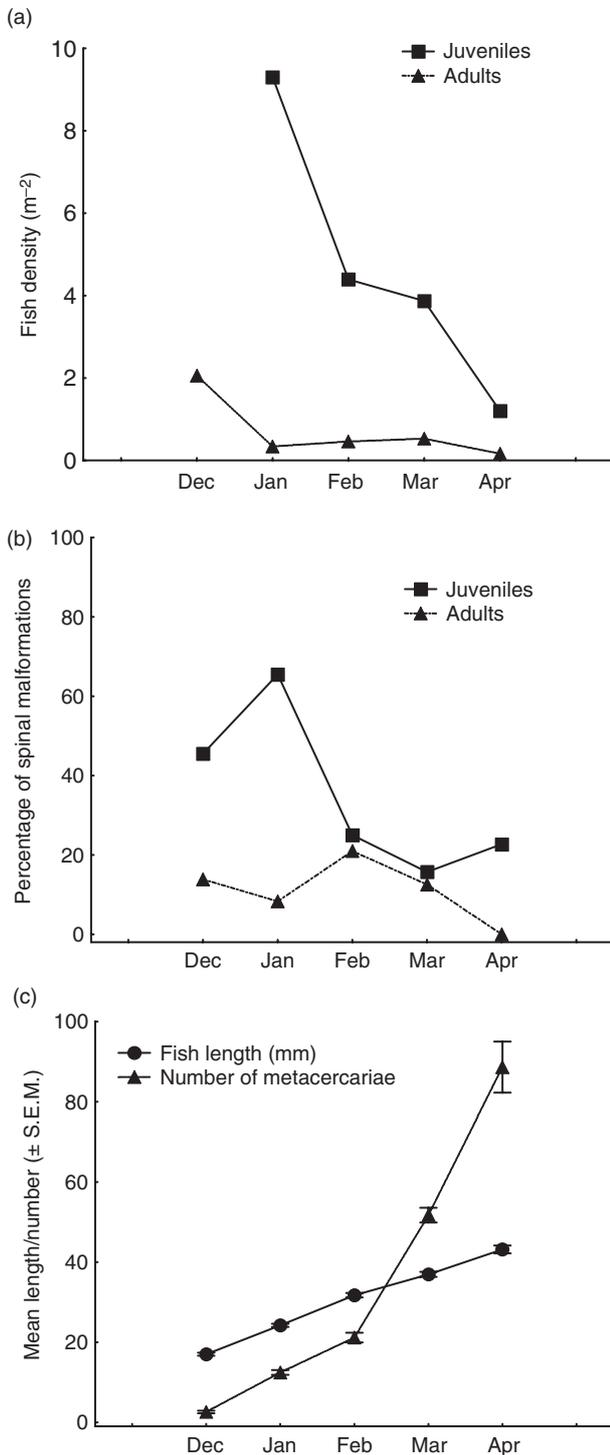


Fig. 3. Temporal changes in (a) fish density, and (b) the frequency of spinal malformation of juvenile and adult *Galaxias anomalus*, and (c) fish length and mean abundance of *Telogaster opisthorchis* metacercariae in juvenile *Galaxias anomalus*. Note that the number of adults sampled per month was as follows: Dec. $n = 43$; Jan. $n = 12$; Feb. $n = 14$; Mar $n = 16$; Apr $n = 5$.

Both snail infection status ($F_{1,28} = 327.6$, $P < 0.0001$), and its interaction with snail abundance ($F_{1,28} = 10.1$, $P < 0.01$), were also significantly related to the severity of spinal malformation (Fig. 5b). While uninfected fish devel-

Table 1. Frequency (%) of fin malformations in juvenile *Galaxias anomalus*

	n	Extra fins	Size asymmetry	Asymmetrically opposed	Infection prevalence (%)
Dec	55	0	0	0	85
Jan	116	8.6	1.7	0.9	100
Feb	68	4.4	0	0	100
Mar	70	1.4	0	0	100
Apr	22	9.0	0	0	100

oped no spinal malformations, such malformations developed in 81.2% and 93.7% of the fish in the low and high infection intensity groups, respectively, with fish developing more severe malformations when exposed to high vs. low infection intensities. Two fish had fin malformations, from different replicates of the low infection intensity group; one had an additional pelvic fin and the other exhibited pelvic fin size asymmetry, with metacercariae observed close to the area of fin insertion to the body in both fish. Snail infection status was also significantly related to fish survival ($F_{1,28} = 17.6$, $P < 0.0001$, Fig. 5c), with fish exposed to infection having lower overall survival than uninfected fish.

Discussion

Previous studies have indicated that, as with amphibians, fish skeletal malformation may be caused by trematode infections (e.g. Cunningham *et al.* 2005; Villeneuve *et al.* 2005). Our study supports this contention, demonstrating that infection by the common New Zealand trematode *T. opisthorchis* is a cause of spinal malformation in the non-migratory fish *G. anomalus*, and that increasing infection intensity increases the extent of spinal malformation, corroborating field patterns. The experiment was conducted at the same time as field sampling, with the natural shedding of cercaria by snails closely matching the levels of infection experienced by fish in the field. Furthermore, our results indicate that the mechanism causing malformations in *G. anomalus* operates during a 'critical window' of development corresponding to the late larval and early juvenile phase. The frequency of spinal malformation in the field increased to a peak from December to January (early summer in New Zealand), most likely due to the lag required for fish developmental responses to infection. However, between January and February, frequency declined sharply and remained low through to April. In addition, a positive relationship between infection intensity and the severity of spinal malformation occurred only in December and January. The lack of relationship from February onwards can be explained by the larger fish present continuing to accumulate metacercariae without infection causing malformations (see Figs 3c and 4c–e). It should be noted that the scatter of points in February and March (Fig. 4c,d) suggests the possibility of two relationships; a smaller set of points indicative of a positive relationship between infection and malformation, and the majority of points showing a flat and non-significant relationship. Subsequent examination of

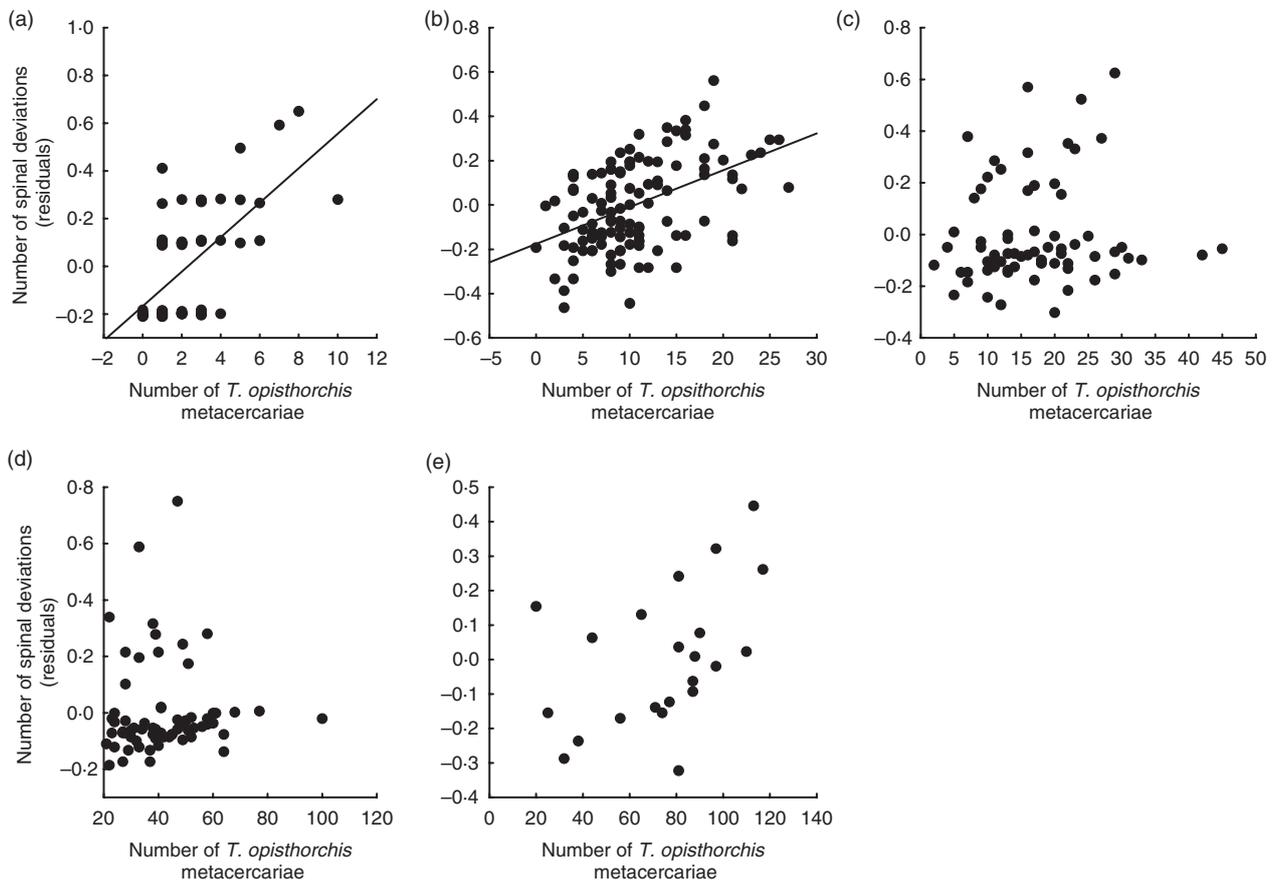


Fig. 4. Relationship between spinal malformation in juvenile *Galaxias anomalus* and infection abundance of *Telostyleria opisthorchis* in, (a) December 2008; (b) January 2009; (c) February 2009; (d) March 2009 (e) April 2009.

size-frequency distributions of fish ruled out the possibility that the pattern was caused by two fish cohorts, each differing in susceptibility to infection. The most likely explanation for the pattern is that fish vary in the site of metacercarial encystment; some fish become infected in areas critical for spinal development whereas others acquire infections in less critical areas but continue to accumulate infections as they grow, without becoming malformed. That susceptibility to trematode induced malformation decreases with fish size is further indicated by the much lower frequency of spinal malformations in adult than juvenile fish.

There is good evidence that trematode metacercariae in amphibians physically impair developing tissue (Sessions & Ruth 1990), with *Ribeiroia*-induced limb malformations being more likely if cercariae encyst during the window of early limb-bud development (Blaustein & Johnson 2003). A similar mechanism is the most probable cause of the spinal malformations in post-larval *G. anomalus* observed here. Indeed, the critical window in which malformations were observed to occur equates to the developing galaxiid length (averages of 17 and 24 mm in December and January respectively; see Fig. 3c) at which the change from cartilaginous to full skeletal ossification occurs (Benzie 1968). That we observed increasingly severe spinal malformations and elevated mortality with increasing infection intensity likely

reflects the greater probability that metacercariae encyst in tissue crucial for spinal development.

Although malformations have been heralded as indicators of a new emerging disease threat to populations (e.g. Blaustein & Johnson 2003; Johnson & Sutherland 2003), evidence that the phenomenon causes population change is lacking. In this study, the sharp decline in the frequency of spinal malformation observed in the field coincided with the period of steepest decline in juvenile densities (9.4 m^{-2} in January to 4.4 m^{-2} in February). While the changes could partly result from density dependence, the pattern strongly suggests that more abnormal juveniles were lost from the population, contributing to the decline. Indeed, the laboratory experiment demonstrates how increasing infection intensities were the likely cause of both malformation and mortality in *G. anomalus* during the critical window. Whether malformed fish died directly as a result of pathology or were preferentially captured by predators is unknown, but the associated experiment indicates that direct trematode-induced mortality would have at least been a factor at this time. While starvation and predation are generally considered to be the main mortality factors impacting fish early life stages and hence determining population recruitment (e.g. Cushing 1996), our results indicate that parasitism may also play a key role. The occurrence of critical windows of susceptibility during fish

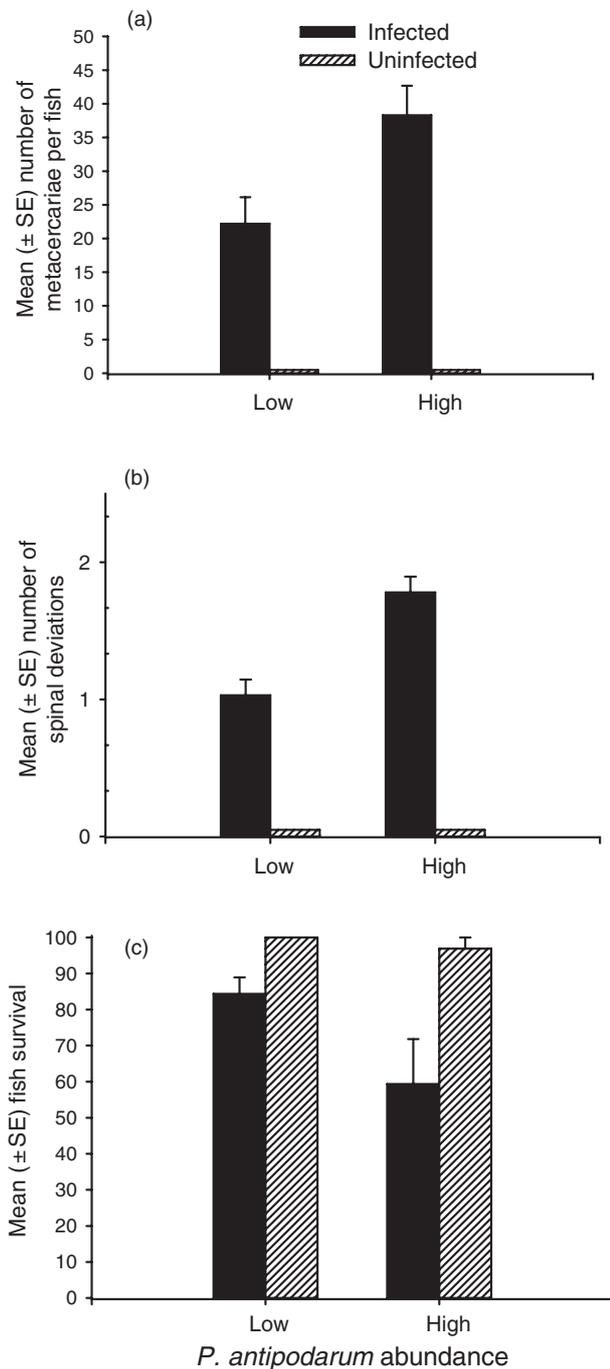


Fig. 5. Laboratory infections of juvenile *Galaxias anomalus* in relation to infection status and abundance of *Potomopyrgus antipodarum*. (a) Infection intensity of *Telogaster opisthorchis* in *G. anomalus*. (b) Extent of spinal malformations in *G. anomalus*. (c) Survival of *G. anomalus*.

development may explain why this mechanism of trematode impact on fish populations has been previously overlooked. For example, based on our data, failure to survey *G. anomalus* in the first 7–8 weeks post-emergence would miss the trematode impacts. However, it should be noted that seasonal studies such as the current one may be too short to address whether patterns observed have long-term population consequences.

Whether *T. opisthorchis* is responsible for malformations occurring in other *Galaxias* species and sites is unknown. For example, Allibone (2000) reported severe head and jaw malformations in *G. depressiceps* and *G. spp. D.*, which we did not observe in *G. anomalus*. While fin malformations were common in *G. anomalus* in the field, there was no experimental support for infection being a cause. As studies have linked malformations in aquatic taxa with contaminants (Rohr *et al.* 2008; Hu *et al.* 2009), we cannot rule out a role of additional stressors. This possibility raises a related concern; many of the environmental stressors purported to increase susceptibility to trematode infections and malformations in wetlands (e.g. eutrophication and pesticide application, Kiesecker 2002; Johnson *et al.* 2007; Rohr *et al.* 2008) are key issues threatening river and lake habitats (e.g. Allan & Flecker 1993). Such stressors may also influence infection in fish via effects on parasite intermediate hosts. For example, in New Zealand streams densities of the snail *P. antipodarum* (the snail intermediate host of *T. opisthorchis*) increase with land-use intensification (Matthaei *et al.* 2006); the unprecedented increases in agricultural intensification in New Zealand (e.g. MacLeod & Moller 2006) could thus affect trematode parasitism. Water abstraction forces galaxiids into poor habitats with low flow, high temperatures, and low oxygen regimes, which compromise juvenile development (Allibone 2000; Leprieur *et al.* 2006). These marginal habitats could exacerbate disease effects by concentrating snail intermediate hosts, cercariae and fish (Mitchell *et al.* 2000), and enhancing cercarial shedding rates (Poulin 2006). Interestingly, Allibone (2000) reported that water abstraction was associated with poor larval growth, a high percentage of malformed adult fish, and high snail densities.

These issues highlight a clear need to further investigate the interactive effects of trematode parasitism and other environmental stressors on animal populations. Because studies of trematode-induced malformations have often been inconclusive (e.g. Skelly *et al.* 2007), a call for a more comprehensive appraisal of the factors influencing the phenomenon (e.g. Johnson & Sutherland 2003) seems valid.

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