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Multiple marking of otoliths of brown trout, *Salmo trutta* L., with alizarin redS to compare efficiency of stocking of three early life stages

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Abstract This study examined stocking with three early life stages of brown trout, *Salmo trutta* L., in the context of rehabilitating a native lineage. Experimental sections in five streams were stocked successively with three stages (I: unfed fry at the end of the reabsorption phase, II: fed fry measuring 2–3 cm and III: fed fry measuring 4–5 cm) derived from a hatchery stock bred from wild spawners. The three stages were distinguished by single or multiple fluoromarking of the otoliths with alizarin redS. The index of relative stocking efficiency was greater for stage II than for stage I in all sections and equivalent or greater than that for stage III. Stage II achieved significantly larger mean length and weight in autumn than stage III stockings.

KEYWORDS: alizarin redS, early life stages, otolith marking, rehabilitation, stocking.

Introduction

Concern about the conservation and rehabilitation of native populations of salmonids is growing (Dodson, Gibson, Cunjak, Friedland, Garcia de Leaniz, Gross, Newburym, Nielsen, Power & Roy 1998; Crivelli, Poizat, Berrebi, Jesensek & Rubin 2000; Hallerman 2003; Caudron, Champigneulle & Guyomard 2006). There is therefore an increasing need for fishery managers to develop operational strategies to rehabilitate native populations. Where the native population is too small or requires support, supplementary stocking is used, with the ultimate goal to support or recreate new functional populations, thus increasing the area of distribution of native lineages. Rehabilitation stocking tends to use juveniles produced from wild stock (supportive breeding) or from captive spawners recently produced from eggs collected from wild native fish (Wang & Ryman 2001; Hallerman 2003).

In this context of rehabilitation of native populations, the restocking method used must meet two conditions: (1) it must make the best possible use of the restocking material that is generally in short supply, which means that it is essential to optimise the effectiveness of the releases and (2) it must restrict the time the fish spend in captivity to limit, as far as possible, the harmful genetic effects linked to domestication (Busack & Currens 1995; Waples 1999). Furthermore, the choice of which stage to release is particularly important as it can have a long-term impact on the survival of the introduced fish (Letcher, Dubreuil, O'Donnell, Obedzinski, Griswold & Nislow 2004), and therefore also affect the success of the restocking programmes. These requirements necessitate the need to carry out comparisons of releasing different early life stages, using a marking method that would not result in any post-restocking inter-group bias linked to the marking technique used.

Three early stages are currently used for salmonids: unfed fry at the end of the yolk sac reabsorption phase, small fed fry measuring 2–3 cm that have been feeding for a few weeks and larger fed fry measuring 4–5 cm. Such stocking procedures have thus far undergone little comparative study (Lasenby & Kerr 2001). The development of otolith fluoromarking techniques has now made it possible to track salmonids restocked at

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different early stages (Champigneulle & Cachera 2003; Caudron & Champigneulle 2006).

The present study used the technique of single and multiple otolith markings using alizarin red S (ARS). This technique was used to compare brown trout, *Salmo trutta* L., restockings at three early life stages (yolk sack fry, fed fry, fingerlings measuring 4–5 cm).

Materials and methods

Sites investigated and stocking characteristics

Juveniles were produced from a single pool of eggs obtained from a captive breeding stock of wild native spawners of the Mediterranean Lineage (ML) caught in the upper part of the Dranse d'Abondance, a French tributary of Lake Geneva. The upper Dranse d'Abondance river has a native ML population of brown trout with a low level of introgression by the Atlantic lineage (Largiadèr, Scholl & Guyomard 1996). Only 98 families obtained from parents (i.e. 98 females and 98 males) showing Mediterranean genotypes at three diagnostic markers, Str541, Str591 and Str791 (Estoup, Largiadèr, Cornuet, Gharbi, Presa & Guyomard 2000) were kept and used (C.R. Largiader, unpublished data).

Five different streams were selected in or at the edge of the area of distribution of the native ML population from which the hatchery stock was bred. Those rivers were representative of the fast-flowing, middle-altitude streams of this territory. The sections into which the experimental releases were carried out were limited upstream and downstream by obstacles that juveniles would find difficult to bypass in an upstream direction. In addition, the sections chosen had low natural recruitment.

Each section (Table 1) was stocked successively with the three different stages (I: unfed fry at the end of the reabsorption phase stocked on 18 and 20 April 2006, II: fed fry measuring 2–3 cm stocked on 26 and 28 May and III: fed fry measuring 4–5 cm stocked on 28 June). Considerable care was taken about the release phase: i.e. choice of appropriate hydrological conditions and ensuring good dispersal of the fry over the study sites. The restocking densities were equivalent on each section, and similar to those used locally, i.e. 1.3–1.4 individuals per m^2 for stages I and II and 0.5–0.6 individuals per m^2 for stage III.

Marking techniques

Each marking involved immersion for 3 h in a bath containing 100 mg L^{-1} of alizarin RedS (ARS) (following Caudron & Champigneulle 2006). No mortality was attributable to the marking. Three types of marking were used to differentiate between the restocking stages. Stage I release – a single ARS marking at the beginning of the reabsorption period at 10 degree-days (°D) after the eggs had hatched. Stage II release – two ARS markings, the first carried out at the beginning of the reabsorption phase and the second at the end of this phase at 10 and 220 °D, respectively, after hatching. Stage III release – three ARS markings, two of which were carried out at the beginning and end of the reabsorption period, as described above, plus one carried out using fed fry or fingerlings measuring 3–4 cm. A control group subjected to the latter marking procedure was used to assess the success of the three marking immersions carried out.

Sample collection

A representative sample of juvenile trout was caught by electric fishing at the end of October 2006 on each of the five stocked sections. For each section, about 120 juveniles were randomly sampled and killed by administering an overdose of an anaesthetic (clove oil), and kept in the freezer at −18 °C until analysis.

The age of each individual was estimated from scales so that only the 2006 cohort was included in the

Table 1. Characteristics of the sections stocked in the five rivers

	Length (m)	Mean width (m)	Area (m^2)	Elevation (m)	Stocking quantities			Sample				
					I	II	III	N	I	II	III	Total
Pamphiot	600	1.5	900	800	1250	1250	500	35	2	39	12	88
Ubine	900	1.2	1080	1300	1500	1500	600	37	1	60	14	112
Combes	600	0.9	540	600	750	750	300	6	0	78	21	105
Masses	500	1.5	750	1200	1000	1000	400	37	0	31	29	97
Moises	500	1.5	750	600	1000	1000	400	13	24	51	18	106

Quantities released at the different stages were distinguished by alizarin red S otolith fluoromarking (I: yolk sac fry at the end of reabsorption; II: 2- to 3-cm fed fry; III: 4- to 5-cm fed fry). Sampling of 0+ fingerlings in October were classified according to the source of recruitment (N = natural recruitment).

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analysis. The head of each individual was dissected to remove the otoliths (sagittae). These otoliths were stuck onto a thin glass slide using a thermo-adhesive (Crystalbond Arenco Adhesive number 509) and polished to expose the core. To find out whether the otolith had been marked, each slide was viewed under an epifluorescence microscope (Zeiss Axioskop 40) fitted with a mercury vapour lamp (HBO50) and an Alizarin filter (Zeiss no. 15: BP546/12, FT 580, LP 590). All the otoliths of the individuals sampled from the natural environment and those from the control group were examined by two observers to determine the reliability of the ARS marking. Each observer classified the otoliths as belonging to one of four categories: no ARS marking (=individuals derived from natural recruitment, scored O), marking patterns I, II and III.

For each site, an index of relative efficiency (IRE) was calculated for each of the three restocking methods by relating the number of 0+ of a restocking method found in October, and the number of fry of the same restocking method released into the section examined. This ratio was only used relatively in the same site to compare the different stocking practices. For a given section, chi-squared tests were used to determine differences between the different stocking methods.

The morphological characteristics (length, mm and weight, g) of the three or four sources of recruitment (O, I, II and III) were compared for each section using Student's *t*-test, and adjusting the *P*-values using the Bonferroni correction (Miller 1981; Trippel & Hubert 1990).

Results

Fifty 0+ individuals derived from the control group with triple marking were examined at the end of October 2006. The three marks were distinct (Fig. 1) on otolith sagittae of all individuals examined. This preliminary validation confirmed that it was possible to distinguish correctly between the different stocking stages.

Index of relative efficiency I was significantly ($P < 0.01$) lower for each of the five sites than both

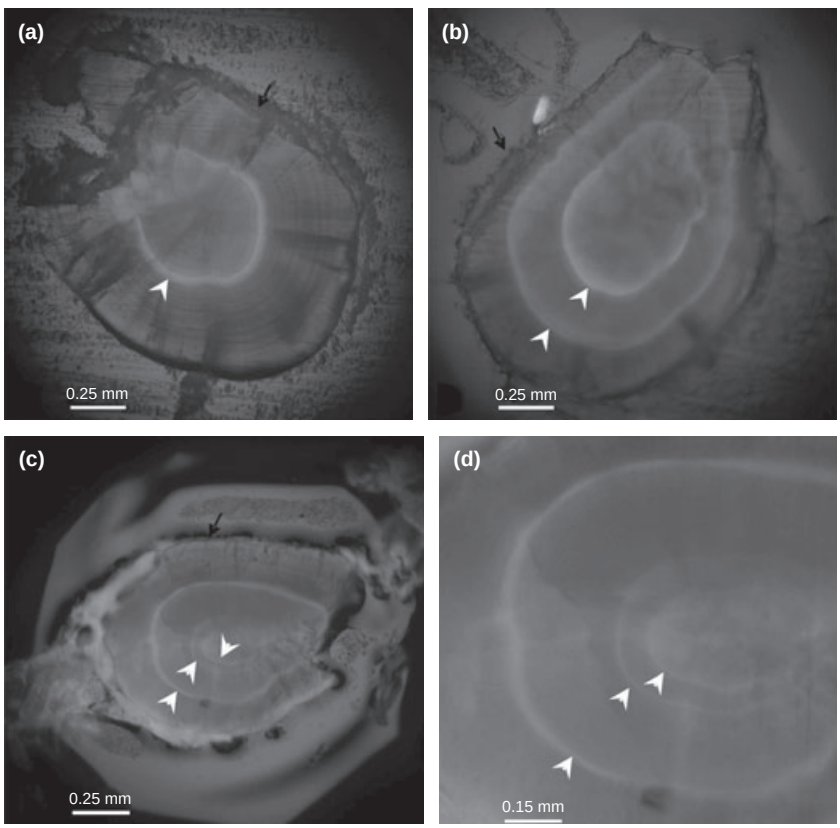

Figure 1. Photographs of polished otoliths (sagittae) of 0+ trout (in October) submitted to one (a: beginning of yolk sac reabsorption), two (b: beginning and end of reabsorption) or three (c and d: beginning and end of reabsorption and at the 2- to 3-cm fed fry stage) 3-h immersion bath containing 100 mg L^{-1} of alizarin red S. White arrows: marking rings, black arrows: the otolith outline.

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Table 2. Index of relative stocking efficiency (%, with 95% confidence limits in brackets)

	Index of relative stocking efficiency (%)		
	I	II	III
Pamphiot	0.2a (0.0–0.7)	3. 1b (2.6–4.9)	2.4b (1.2–4.0)
Ubine	0.1a (0.0–0.3)	4.0b (2.7–4.5)	2.3b (1.5–4.5)
Combes	0.0a (0.0–0.5)	10.4b (7.4–11.3)	7.0b (5.7–13.7)
Masses	0.0a (0.0–0.5)	3.1b (1.9–4.0)	7.2c (3.9–8.0)
Moises	2.4a (1.5–3.4)	5.1b (3.4–5.9)	4.5b (2.1–5.4)

The index was calculated as a % of the ratio between the number of 0+ fish in October and the number originally released for each of the stocking methods used. (I: yolk sac fry at the end of reabsorption; II: 2- to 3-cm fed fry; III: 4- to 5-cm fed fry). For a given river, indices of relative efficiency followed by different letters are significantly different ($P < 0.05$).

IRE II and IRE III (Table 2). At four of five sites, there was no significant ($P > 0.05$) difference between IRE II and IRE III (Table 2). At the fifth site (the Masses stream), IRE III was significantly ($P < 0.01$) higher than IRE II (Table 2).

In all five sections, natural recruitment generally accounted for a minority of recruitment, with contribution ranging from 6% to 40% of the 0+ autumn population (Table 1). For each section, the contribution of stage II was significantly ($P < 0.01$) higher than that of stage I, which made a very small contribution (0–2%) at four of five sections. In four of five sections, the contribution of stage II was also greater than the contribution of stage III. Only the Masses stream displayed similar contributions ($P > 0.05$) between stages II and III.

The numerical contributions of the various stages determined in October display major changes from their numerical contributions to the release (Fig. 2). In four of five sections, the autumn contribution of stage I was significantly ($P < 0.01$) lower than the initial contribution. At the fifth site (Moises river), the contributions did not significantly differ ($P < 0.01$). The contribution of the stage II release was significantly ($P < 0.01$) higher at all five sites in October than its initial contribution. For stage III, the initial contributions and those observed in October were comparable ($P < 0.05$) in four sections, whereas it increased in one section (Masses stream).

At the beginning of October, in all the five sections, fingerlings stocked at stage II displayed significantly higher mean lengths and weights ($P < 0.05$) than fry stocked at stage III (Fig. 3). At three of five sites, the individuals stocked at stage II displayed mean lengths and weights in October that were significantly $P < 0.05$) higher than that of individuals derived from natural recruitment (Fig. 3). In the Moises and Pamphiot sections, the wild individuals and individuals

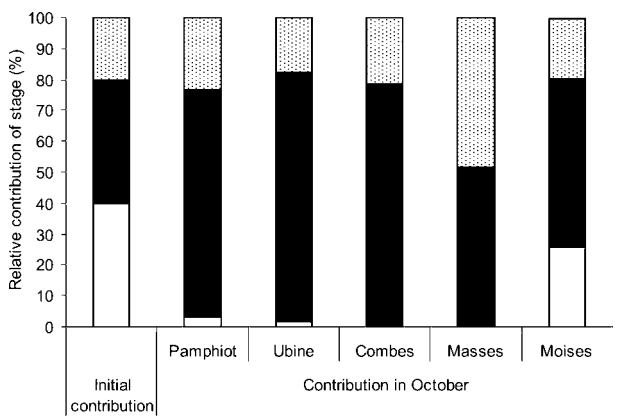

Figure 2. Relative contribution at stocking and in 0+ stage in October for three successive stocking operations (white: stage I; black: stage II; dotted: stage III; in sections of five rivers).

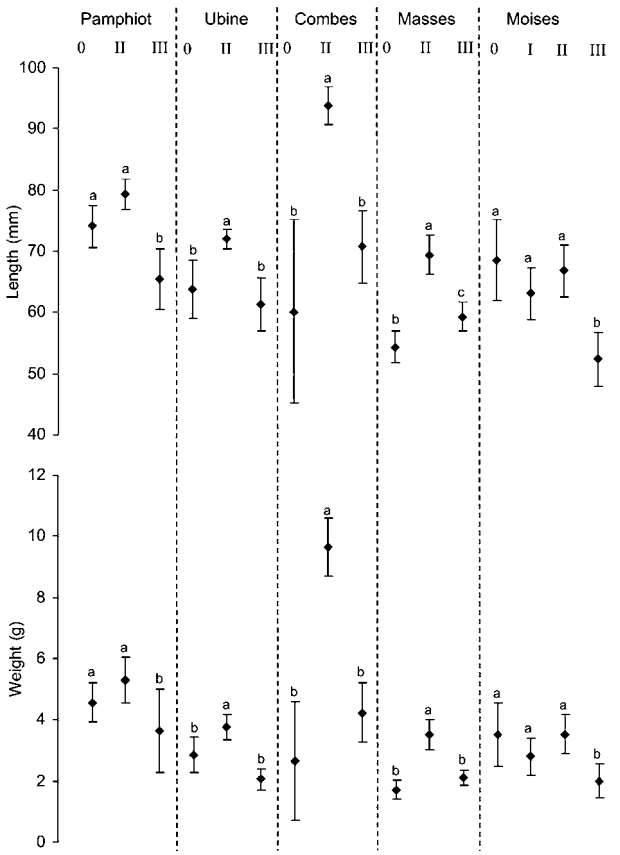

Figure 3. Total length (mm) and weight (g) (mean and 95% confidence limits) of 0+ trout in October according to origin. (N = natural; I, II, III – stocking stages I: yolk sac fry at the end of reabsorption; II: 2- to 3-cm fed fry; III: 4- to 5-cm fed fry). When two mean values have different letters, they are significantly different for a given river ($P < 0.05$).

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stocked at stage II had the same ($P < 0.05$) mean length and weight in October.

Discussion

Possibilities of ARS marking

The findings confirmed the observation of Caudron & Champigneulle (2006) that a 3-h bath in a solution of 100 mg L^{-1} ARS carried out at three different early life stages (beginning, end of reabsorption and 3–4 cm) can be used to distinguish at least three different groups from the earliest fry stages without ambiguity by means of direct epifluorescence microscopic observation. The present study further demonstrated the possibility of using a third immersion at the 3- to 4-cm fry stage to produce a distinguishable third marking ring. This ring can be observed at the same time as the first two rings, in the same otolith polishing plain. Thus, direct epifluorescence microscopic observation (without measurement of the ARS rings) provides an easy way to distinguish between the single-, double- and triple-marked groups.

Comparative effectiveness of restocking with early life stages

The choice of a restocking strategy or practice must take into account the purpose and objectives of the restocking operation (Cowx 1994; Waples 1999; Crivelli *et al*. 2000). In the context of rehabilitating native populations by using local native strains, there is a need to stock at early life stages to limit harmful genetic effects linked to domestication (Ryman & Laikre 1991; Busack & Currens 1995; Waples 1999). However, the restocking material is generally available in low quantities; so, it is necessary to optimise the stocking efficiency at early life stages, a topic that is still poorly studied (Lasenby & Kerr 2001; Letcher *et al*. 2004), although these practices are currently used for salmonids (Cowx 1994; Brown & Day 2002; Mobrand, Barr, Blankenship, Campton, Evelyn, Flagg, Mahnken, Seeb, Seidel & Smoker 2005).

The present study showed that stocking success with yolk sac fry tended to be very low and was even virtually zero at four of the five studied sections. By contrast, releases carried out at the same densities but with fry that had begun to feed (stage II) were much more efficient. This better efficiency was obtained despite stage I individuals having the advantage of prior residence. This could be partly attributable to the yolk sac fry being sensitive to extreme hydrological conditions. In the zone of present study, heavy rainfall was observed in March (149 mm) and April (143 mm) 2006. Cattaneo, Lamouroux, Breil & Capra (2002) showed that the density of 0+ trout was strongly and inversely related to the rate of flow during the hatching period. On the other hand, stage II fry that were fed for 6 weeks in the hatchery avoided both spates and the critical phase of the first *in situ* feed, which is recognised as being a phase associated with high mortality in trout (Elliott 1994).

Stage II releases exhibited similar or even better relative efficiencies than those of fry released 1 month later and when they were bigger (stage III). This was possibly because individuals stocked at stage II colonised habitats that were not yet saturated, which gave them an advantage linked to their prior residence over individuals introduced later at stage III. Several authors demonstrated the competitive advantage of prior residence for salmonids (Glova & Field-Dodgson 1995; Huntingford & De Leaniz 1997; Deverill, Adams & Bean 1999). Letcher *et al*. (2004) also reported that prior residence in the Atlantic salmon allows the individuals that are introduced earlier to reach a larger size.

The results suggest that releases of stage I were too inefficient and/or variable in a context of limited egg quantities and releases of stage III offered no clear advantage over that of stage II. As both stages II and III allow choice over optimal hydrological conditions at the time of stocking but the stay in the hatchery is smaller for stage II than for stage III, it appears advantageous to stock stage II. In addition, stage II fry tend to have autumnal morphological characteristics that could also be advantageous in terms of winter survival (Cunjak 1998). However, the present study is only exploratory in nature. It would be necessary to develop further experiments so that the stocked fish do not compete in the experimental areas. The initial density of wild populations in the stocked areas should also be taken into account. It would also be useful to extend the monitoring beyond the zones where the experimental releases were performed to get an overall picture about the mobile fractions of stocked fry. There is also a need to evaluate over the longer survival of the different life stages stocked.

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References

Brown C. & Day R. (2002) The future of stock enhancement: lessons for hatchery practice from conservation biology. *Fish and Fisheries* **3,** 79–94.

Busack C.A. & Currens K.P. (1995) Genetic risks and hazards in hatchery operations: fundamental concepts and issues. *American Fisheries Society Symposium* **15,** 71–80.

Cattaneo F., Lamouroux N., Breil P. & Capra H. (2002) The influence of hydrological and biotic processes on brown trout (*Salmo trutta*) population dynamics. *Canadian Journal of Fisheries and Aquatic Sciences* **59,** 12–22.

Caudron A. & Champigneulle A. (2006) Technique de fluoromarquage en masse à grande échelle des otolithes d'alevins vésiculés de truite commune (*Salmo trutta* L.) à l'aide de l'Alizarine red S. *Cybium* **30,** 65–72 (In French).

Caudron A., Champigneulle A. & Guyomard R. (2006) Assessment of restocking as a strategy for rehabilitating a native population of brown trout (*Salmo trutta* L.) in a fast-flowing mountain stream in the northern French Alps. *Journal of Fish Biology* **69,** 127–139.

Champigneulle A. & Cachera S. (2003) Evaluation of large-scale stocking of early stages of brown trout, *Salmo trutta*, to angler catches in the French-Swiss part of the River Doubs. *Fisheries Management and Ecology* **10,** 79–85.

Cowx I.G. (1994) Stocking strategies. *Fisheries Management and Ecology* **1,** 15–30.

Crivelli A., Poizat G., Berrebi P., Jesensek D. & Rubin J.F. (2000) Conservation biology applied to fish: the example of a project for rehabilitating the marble trout (*Salmo marmoratus*) in Slovenia. *Cybium* **24,** 211–230.

Cunjak R.A. (1998) Physiological consequences of overwintering in streams: the cost of acclimatization? *Canadian Journal of Fisheries and Aquatic Sciences* **45,** 443–452.

Deverill J.I., Adams C.E. & Bean C.W. (1999) Prior residence, aggression and territory acquisition in hatchery-reared and wild brown trout. *Journal of Fish Biology* **55,** 868–875.

Dodson J.J., Gibson R.J., Cunjak R.A., Friedland K.D., Garcia de Leaniz C., Gross M.R., Newburym R., Nielsen J.L., Power M.E. & Roy S. (1998) Elements in the development of conservation plans for Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences* **55,** 312–323.

Elliott J.M. (1994) *Quantitative Ecology and the Brown Trout*. Oxford: Oxford University Press, 286 pp.

Estoup A., Largiadèr C.R., Cornuet J.M., Gharbi K., Presa P. & Guyomard R. (2000) Juxtaposed microsatellite systems as diagnostic markers for admixture: an empirical evaluation with brown trout (*Salmo trutta L.*) as model organism. *Molecular Ecology* **9,** 1873–1886.

Glova G.J. & Field-Dodgson M.S. (1995) Behavioural interactions between chinook salmon and brown trout juveniles in a simulated stream. *Transactions of American Fisheries Society* **124,** 194–206.

Hallerman E.M. (2003) *Population Genetics and Applications for Fisheries Scientists*. Bethesda, MD: American Fisheries Society, 458 pp.

Huntingford F.A. & De Leaniz C.G. (1997) Social dominance, prior residence and the acquisition of profitable feeding sites in juvenile Atlantic salmon. *Journal of Fish Biology* **51,** 1009–1014.

Largiadèr C.R., Scholl A. & Guyomard R. (1996) The role of natural and artificial propagation on the genetic diversity of brown trout (*Salmo trutta* L.) of the upper Rhône drainage. In: A. Kirchhofer & D. Hefti (eds) *Conservation of Endangered Freshwater Fish in Europe*. Basel: Birkhauser Verlag, pp. 181–197.

Lasenby T.A. & Kerr S.J. (2001) *Brown Trout Socking: An Annotated Bibliography and Literature Review*. Peterborough: Fish and Wildlife Branch, Ontario Ministry of Natural Resources, 196 pp.

Letcher B., Dubreuil T., O'Donnell M., Obedzinski M., Griswold K. & Nislow K. (2004) Long-term consequences of variation in timing and manner of fry introduction on juvenile Atlantic salmon (*Salmo salar*) growth, survival, and life-history expression. *Canadian Journal of Fisheries and Aquatic Sciences* **61,** 2288–2301.

Miller R.G. (1981) *Simultaneous Statistical Inference*. New York: McGraw Hill, 299 pp.

Mobrand L., Barr J., Blankenship L., Campton D., Evelyn T., Flagg T., Mahnken C., Seeb L., Seidel P. & Smoker W. (2005) Hatchery reform in Washington State: principles and emerging issues. *Fisheries* **30,** 11–23.

Ryman N. & Laikre L. (1991) Effects of supportive breeding on the genetically effective population size. *Conservation Biology* **5,** 325–329.

Trippel E. & Hubert J. (1990) Common statistical errors in fishery research. In: J. Hunter (ed.) *Writing for Fisheries Journals*. Bethseda, MD: American Fisheries Society, pp. 93–102.

Wang J. & Ryman N. (2001) Genetics effects of multiple generations of supportive breeding. *Conservation Biology* **15,** 1619–1634.

Waples R.S. (1999) Dispelling some myths about hatcheries. *Fisheries* **24,** 12–21.

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