Hybridisation between *Pseudorasbora parva* and *Leucaspius delineatus*

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Received 9 May 2005; Accepted 29 September 2005

A b s t r a c t . Hybridisation between introduced and native species is an increasingly important issue concerning environmental stakeholders because it decreases genetic integrity of native stocks. However, hybridisation could also happen between non-native species even if these species belong to different genera. Our study illustrates this particular aspect of alien species invasion with the attempt of artificial hybridisation between sunbleak *Leucaspius delineatus* and topmouth gudgeon *Pseudorasbora parva,* both recent additions to the freshwater fish fauna of England. The crossed fertilisation of *L. delineatus* ova with *P. parva* sperm lead to viable eggs with a very high hatching rate of 86% although hybrids failed to develop beyond the 1st larval step. Early morphological development of these hybrids was quantified and compared to the early development of the pure bred species.

Key words: early life history, morphology, non-native, topmouth gudgeon, sunbleak

Introduction

Human travel and trading practices have effectively eliminated biological isolation and species which used to be allopatric become geographically sympatric within a short time. These former allopatric species presumably relied on geographic isolation rather than reproductive barriers for their speciation. Therefore, many allopatric species with strong morphological divergence could have the capacity to hybridise. Previous cases of interbreeding between cyprinids like roach *Rutilus rutilus* and bream *Abramis brama* (W h e e l e r 1969, D i a m o n d 1985) and between crucian carp *Carassius carassius* and goldfish *Carassius auratus* (W h e e l e r 2000) has drawn increasing attention to the possible issues associated with the introduction of non-native cyprinids. The present study examines the compatibility of the gametes of sunbleak *Leucaspius delineatus* and topmouth gudgeon *Pseudorasbora parva* (Temminck & Schlegel) and compares the embryonic and early larval development of the experimentally induced hybrids to the pure bred species (P i n d e r & G o z l a n 2004). *P. parva* is a small cyprinid, originating from Japan, China, Korea and the River Amur catchment. Since its inadvertent introduction into Romanian ponds, neighbouring the lower course of the River Danube in 1960 and its successful reproduction in 1961 (B ă n ă r e s c u 1964), *P. parva* has rapidly spread across continental Europe. In contrast, *L. delineatus,* the only representative of this genus and the only nest guarding species among European cyprinids, is a small fish native to continental Europe. Once widespread in Europe, in the last 40 years *L. delineatus* has inexplicably declined and is now on the European list of threatened freshwater fishes (L e l e k 1987). Large number eggs of both species have already been found regularly on stems of water lilies (Centre for Ecology & Hydrology unpublished data). In contrast to *L. delineatus*, *P. parva* already has a history of hybridisation with another species of the same genus, *Pseudorasbora pumila pumila* (Miyadi), which is now threatened by extinction in Japan (K o n i s h i et al. 2003).

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Materials and Methods

On May 24th 2004 eggs were acquired from three *L. delineatus* females (mean ± S.E., L_F = 60.67 ± 2.85 mm) by gently stroking their abdomens. This was in order to describe the early ontogeny of experimentally induced hybrids from activation for comparison with the pure bred strains (P i n d e r & G o z l a n 2004). The female gametes were mixed in several Petri dishes with the sperm of three *P. parva* males (mean ± S.E., L_F = 64 ± 1.96 mm). The inseminated eggs were spread over the surface of the Petri dishes just before they became sticky. The Petri dishes were then transferred to an 80 l capacity rearing aquarium, fitted with a small air stone to provide aeration and water circulation.

For comparison, pure bred *L. delineatus* and *P. parva* eggs were acquired naturally in tanks from adult broodstock originating from the Stoneham Lakes (NGR: SU 438 173) and Crampmoor fisheries (NGR: SU 38414 22216) both in the South of England. After spawning, the eggs were immediately transferred to separate tanks under the same condition as for the hybrids.

Water temperatures in the tanks were recorded hourly, from activation to the end of the study period, using a 'Tiny talk' recorder. The timescale used against development is presented as accumulated degree days post-activation (a°d), where a°d = sum of mean daily temperatures (°C). Time is presented in days, hours and minutes after activation. Embryonic development, from activation to hatching, was recorded using a binocular microscope, fitted with a phototube and a Leica DC 100 digital camera. This was linked to a computer for the storage of image obtained during early development. After hatching, larvae in the aquarium were fed with *Artemia* sp. (brine shrimp) nauplii complemented with a mixture of ground dried flakes via an automatic feeder.

Samples of fish were taken at regular intervals after hatching. Individuals from each sample were anaesthetized with 2-phenoxyethanol, examined under a binocular microscope and photographed using the Leica DC100 digital camera, before being preserved in 4% formaldehyde solution. Measurements were made to the nearest 0.1 mm using an ocular graticule fitted to the binocular microscope. Lengths are given as notochord length (L_N) for specimens prior to preflexion of the urostyle, and total length (L_T) after flexion. Analysis of the different developmental steps was made on both preserved and living specimens following the developmental model proposed by P i n d e r & G o z l a n (2004) for *L. delineatus*.

Fifteen morphometric characters related to swimming capacity and feeding behaviour were measured on hybrids as well as on pure bred species, following the techniques of H o l č í k (1989) and K o v á č (1992): PrO = pre-orbital distance; PsO = post-orbital distance, Oh = orbital horizontal distance, Ov = orbital vertical distance, Hl = head length, IoD = inter-orbital distance, OoD= Outer-orbital distance, Gape, Mxbh = maximum body depth, Mnbh = minimum body depth, PpD = pre-pectoral distance, PrA = pre-anal distance, PsA = post-anal distance, MxbW = maximum body width, MnbW=minimum body width. One-way ANOVA was performed to compare morphological characters between the hybrids and the pure bred *L. delineatus* and *P. parva*.

Results

Water temperature in the aquarium was constant during the entire length of the study (mean rearing temperature ± S.E., 20 ± 0.0074 °C). Mortality during the different developmental phases in the pure bred species was relatively low, around 10%. However, the overall average mortality of hybrids during the embryo period (i.e. between the subsequent developmental steps) was higher (mean mortality ± S.E., 14 ± 1.15 %) essentially due to the blockage of the

embryonic development of eggs at the start of epiboly (Fig. 1). These eggs remained at the same state until the end of the usual embryo period. The hybrid larvae did not manage to feed consistently even though the mouth was well formed and functional, the swimbladder inflated and some *Artemia* nauplii were found in the intestines during the mixed feeding step. Consequently their overall development slowed down after full absorption of the yolk reserves and no hybrid larvae survived beyond 18 days after activation.

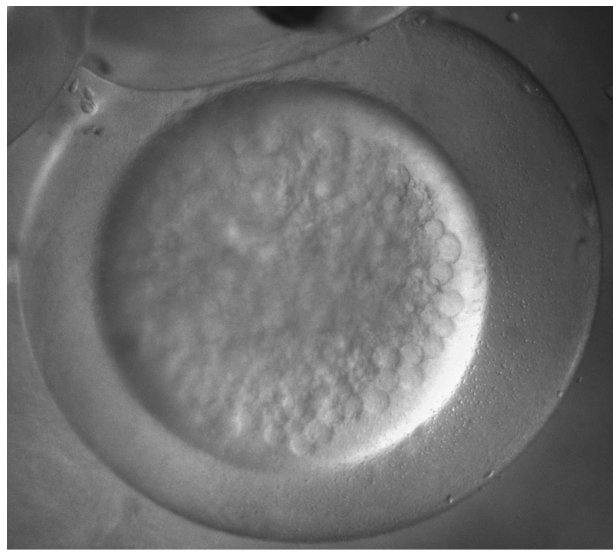

Fig. 1. Epiboly-a stage at which the development stopped in some individuals.

Embryo period

Cleavage phase

Step E1, blastodisc and perivitelline space formation. A few seconds after activation the ova absorbed on water, increasing in volume, creating the perivitelline space and at the same time became adhesive. Ova shape was initially spherical, subsequently becoming slightly oval in shape with a grey-beige colour; the envelope was clear (Fig. 2a). Egg diameter varied between 1.29 and 1.5 mm (mean diameter ± S.E., 1.38 ± 0.0342 mm, n = 7). Blastodisc diameter varied between 82 and 90 percent of egg diameter (mean yolk diameter ± S.E., 86.86 ± 1.03 %, n = 7).

Step E2, cleavage. The cleavage step started at 29 min after activation with the first division of the blastodisc resulting in the formation of blastomeres. Eight blastomeres were clearly visible 1hr and 43 mins after activation (Fig. 2b), and a small celled morula was present 4 h 30 min after activation.

Step E3, epiboly. Epiboly started at 5 h 17 min after activation (17.5 a^{o}d), with the blastoderm spreading over the surface of the yolk. This step ended at 20 h 28 min after activation, with the closure of the germ ring.

Embryo phase

Step E4, organogenesis. At the start of this step the embryonic shield became elongate and cephalisation of the embryo became visible. At 20 h 39 min (36.15 a^{o}d) the embryonic shield

was clearly visible (Fig. 2c) and by 25 h 38 min the optic vesicle first became evident (Fig. 2d). After 42 h 30 min (54.49 a°d) the number of somites was 17, a rudimentary notochord and eye lens were visible, and pigmentation was lacking (Fig. 2e). The shape of the yolksac suggested the onset of its future division into two parts, and the yolk diameter varied between 57 and 62 percent of egg diameter (mean yolk diameter ± S.E., 58.25 ± 1.75 %). By the end of the step cephalisation was evident, with the presence of the olfactory bulb.

Step E5, onset of muscular contraction. The first myotome contraction was evident, with blood circulation, at 67 h 13 min (73.29 a°d). At the start of this step pigmentation was lacking and the blood was colourless. The head was bent down and still attached to the yolksac, which was clearly divided into a round anterior part and a cigar shaped posterior part. The periviteline space allowed the embryos to move freely from time to time by contracting their entire bodies. At 90 h 15 min (93.35 a°d) the auditory vesicles and the olfactory pits were first visible and the blood started to take on a pinkish colour (Fig. 2f). The number of myomeres increased (mean number ± S.E., 36 ± 0.41 %). Pigmentation increased in the eyes but was still lacking on the body, and mesenchyme concentration consisted of two patches on the yolksac close to the embryonic shield, corresponding to the anlagen of the pectoral fins (Fig. 2f). At 97 h 17 min (113.46 a°d) the otoliths were clearly visible, as well as the vascular system with the caudal vein, the ductus cuvieri and brown pigments appeared in the eyes (Fig. 2g).

Free-embryo phase

Step E6, free embryo. This step started at 147 h 12 min (115.78 a°d) with the first hatching of the embryos (89% hatching rate) and finished with the onset of exogenous feeding. Blood circulated from the dorsal aorta to the caudal artery, which supplied the caudal vein. The caudal vein consisted of a very thin network of anastomosing vessels (Fig. 3a), which united into a single subintestinal vein near the formative anal region. The anterior vitelline veins, formed by a single vessel (ductus cuvieri) on both hemispheres, joined the anteroventral part of the yolk sac to enter the heart (Fig. 3a).

The head was bent forward over the yolksac, but separated and slightly extended upward. The straightening process continued to the end of this step, but the free embryo was still curved at the end of the step (Fig. 3b). The urostyle was straight and surrounded by a concentration of mesenchyme. The mouth was turned downward but was not yet functional. The pectoral fin had a round shape and was not very mobile. The embryos contained the final number of somites: 22–23 pre-anal & 17–18 post-anal.

Pigmentation of the hybrids was much fainter at hatching with few melanophores restricted to the lower part of the yolk sac and to the medio-ventral part. By the end of this step the pigmentation increased around the head to the heart, the ventro-visceral part of the abdomen, and the lower part of formative post-anal segment (Fig. 3b). The dorsal part of the developing swimbladder also slowly became covered with dark pigment. The free embryos were lying well spaced at the bottom of the tank and did not congregate at any point in time. They did not show a reaction to light (photophobia) but responded to tapping on the side of the tank with sudden vertical darting movements before sinking again to the bottom of the tank.

Larval period

Step L1, onset of exogenous feeding. As soon as the lower jaw were mobile and the mouth functional (169 h 21 min; 156.01 a°d), the fish were able to inflate the posterior chamber of

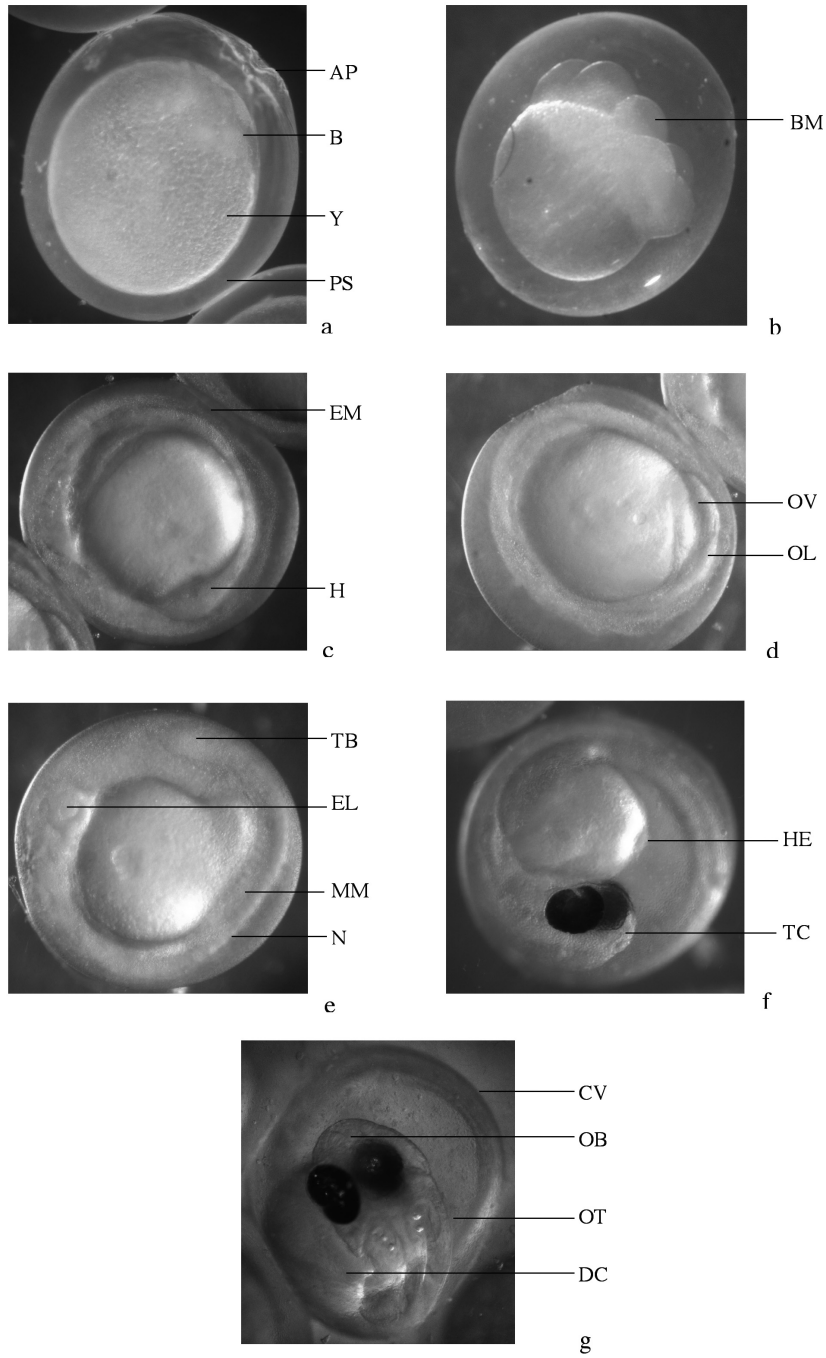

Fig. 2. Embryonic development of the hybrids *L. delineatus* x *P. parva*. (a) activation, min; (b) cleavage phase, step 2, 1 h 43 min; (c) Embryo phase, step 4, 20 h 39 min; (d) Embryo phase, step 4, 25 h 38 min; (e) Embryo phase, step 4, 42 h 30 min; (f) Embryo phase, step 5, 90 h 15 min; (g) Embryo phase, step 5, 97 h 17 min; AP; Animal pole; B, blastodisc; BM, blastomere; CV, caudal vein; DC, ductus Cuvieri; EL, eye lens; EM, embryo; N, notochord; H, head; HE, heart; MM, myomere; OL, optic lobe; OB, olfactory bulb; OT, otholith; OV, optic vesicle; PS, perivitelline space; TB, tail bud; TC telencephalon;. Y, yolk.

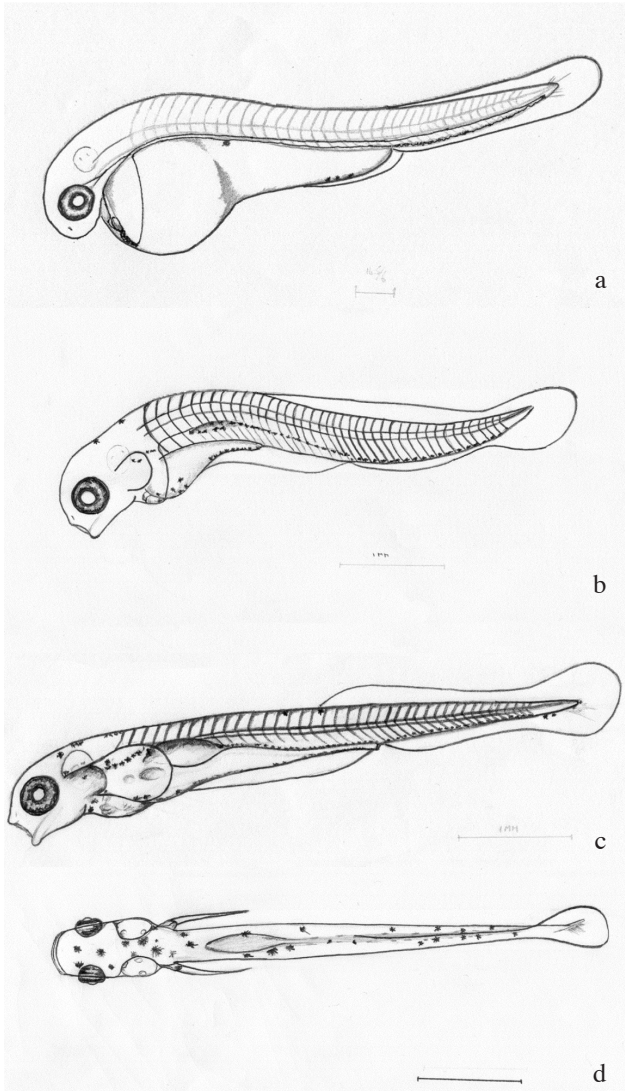

Fig. 3. Developmental steps of hybrid (*L. delineatus* ova with *P. parva* sperm): (a) early free embryo (4.2 mm L_T), (b) late free embryo (4.6 mm L_T), (c) larva 1 lateral view (5.5 mm L_T), (d) larva 1 dorsal view.

the swimbladder and begin to feed exogeneously (Fig. 3c). The mouth was in a subterminal position (mean gape size ± S.E., 64 ± 1.96 mm) and the larvae fed only at the bottom of the tank. Brown melanophores appeared at the dorsal edge of the medulla oblongata (Fig. 3d) and black melanophores increased rapidly, in particular along the subintestinal and caudal vein. The presence of yolk residuals in the larvae, as well as the presence of *Artemia* nauplii in the intestine near the anus suggested mixed feeding. The pectorals fins were vertical and did not appear to be flexible. Some lepidotrichia started to appear in the caudal mesenchyme. The yolk sac disappeared by the end of this step. The larvae did not develop escape behaviour and the last larvae died at 194 h 32 min after activation (281.48 $a^{o}d$).

Few significant morphological differences were found between hybrids and pure bred species including smaller eye diameter, shorter pre-pectoral distance and jaw structure (Table 1, Fig. 3).

Table 1. One way ANOVA of the 17 morphological characters for larva1 hybrids (*L. delineatus* ♀ x *P. parva* ♂) and control (pure bred). * indicates significant differences ($p \leq 0.05$) and ** ($p \leq 0.01$). See text for abbreviations.

	Hybrids		*L. delineatus*		*P. parva*		
Characters	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	***P***
L_T	4.93	0.07	5.91	0.14	5.13	0.84	0.153
L_S	4.55	0.08	5.64	0.12	3.98	1.04	0.011
PrO	0.21	0.10	0.13	0.01	0.24	0.08	0.036
PsO	0.41	0.00	0.47	0.03	0.56	0.15	0,158
Oh	0.35	0.03	0.41	0.01	0.49	0.14	0.159
Ov	0.29	0.04	0.33	0.02	0.42**	0.07	0.002
Hl	0.71	0.10	0.68	0.06	0.84**	0.11	0.007
IoD	0.25	0.02	0.18	0.09	0.24	0.11	0.528
OoD	0.68	0.09	0.88*	0.01	0.81*	0.10	0.026
Gape	0.16	0.00	0.17	0.04	0.14	0.02	0.089
Mxbh	0.73	0.05	0.47	0.07	0.76	0.23	0.047
Mnbh	0.22	0.04	0.13	0.02	0.22	0.09	0.171
PpD	0.87	0.08	0.98	0.08	1.14*	0.20	0.038
PrA	3.16	0.10	3.20	0.09	3.68	0.50	0.056
PsA	2.05	0.07	2.38**	0.12	1.85	0.16	0.000
MxbW	0.47	0.02	0.28	0.02	0.48	0.14	0.022
MnbW	0.19	0.00	0.09**	0.02	0.18	0.04	0.001

Discussion

Our results, provide an interesting insight into the genetic frontiers among fish genus, in particular when biological isolation has been removed. Hybridisation within the genus *Pseudorasbora* has already been reported (K o n i s h i et al. 2003) as well as one experiment of intergeneric hybridisation with *Gnathopogon elongatus elongatus* (K a s a m a & K o b a y a s i 1987). However this is the first attempt of induced intergeneric hybridisation for *L. delineatus*. Previous hybridisation experiments using *Pseudorasbora parva* with other fish species have reported the hybrids to be morphologically intermediate (K a s a m a & K o b a y a s i 1987) to both species strains. However, the morphological characters of our hybrids confirm that phenotypic expression in intergeneric cyprinid hybridisation is not consistently intermediate (R o s s & C a v e n d e r 1981, K a s a m a & K o b a y a s i 1987). Overall, body structure seems consistent with *P. parva* and head characteristics with *L. delineatus*.

The early ontogeny of an organism is a complex sequence of longer stabilized states (steps) with shorter, intermittent less-stable intervals (thresholds). Timing during early ontogeny is crucial for successful development and includes two very important aspects which are synchrony (coordinating) and heterochrony (implementing). K o v á č (2002) suggested that organisms may create their own times for their development. The present study shows that *L. delineatus* eggs could be readily fertilised with *P. parva* sperm and produced hybrids that appeared normal. However, the fact that no larvae survived further than larval step 1 may reflect problems of synchrony and heterochrony as a result of gene expression in inter-generic crosses. If timing in the development of one of the structures or organs related to the transition between larval

step 1 and 2 failed then the young larvae will be unable to survive further. Similar detrimental effects resulting from failure of synchronization have also been observed in many other species (Liem 1991, Langová 1994, Sakakura & Tsukamoto 1999). Failed synchrony could be the mechanism involved in the speciation of sympatric population.

Acknowledgements

The authors would like to thank J. Gozlan who edited with care the various drafts.

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