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swimbladder, significant differences in family size were observed only among half-sibling sire families, whereas in larvae with hyper-inflated swimbladder such differences were found both among half-sibling sire and dam families. The results suggest that paternally and maternally inherited factors may contribute to the expression of swimbladder anomalies in sea bass along with major environmental clues.

Genetic investigation of swimbladder inflation anomalies in the European sea bass, *Dicentrarchus labrax* L.

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Abstract

Although the aetiology of swimbladder inflation anomalies in important aquaculture species such as European sea bass *D. labrax* is not fully determined, culture conditions are commonly suggested as main contributory factors. Little information is available on whether swimbladder inflation has a genetic basis for its expression too. In this work, 24 full-sibling sea bass families from a 4 dams x 6 sires factorial crossing were reared under communal conditions. The larvae developing normal and abnormal (uninflated or hyper-inflated) swimbladders were genotyped at four microsatellite loci, *Labrax-3*, *Labrax-13*, *Labrax-17*, *Labrax-29*, and allocated to the individual breeders. Out of 273 offspring, 97% could be assigned to a single parental pair. The genotype and pedigree analysis showed an imbalance in family size due to differential survival of larvae with normally-inflated swimbladders, with the offspring generated from one dam and one sire being two- to three-fold superior to the other parents, respectively. In larvae with non-inflated swimbladder, significant differences in family size were observed only among half-sibling sire families, whereas in larvae with hyper-inflated swimbladder such differences were found both among half-sibling sire and dam

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Keywords: swimbladder; anomalies; genetics; sea bass; *Dicentrarchus labrax*

1. Introduction

The swimbladder of fishes is a hydrostatic, buoyancy-regulating organ which develops during early larval ontogeny from the dorsal wall of the digestive tract. It also plays a role in the perception and production of sounds as well as in respiratory processes. The swimbladder may inflate either through the transfer of atmospheric air via a pneumatic duct, as in physostomous fish, or by internal gas diffusion like in physoclist fish, or some combination of both (Alexander, 1966; Pelster, 1998). Some physoclist fish, which include the European sea bass (*Dicentrarchus labrax*), are transient physostomous as larvae, possessing a temporary pneumatic duct and seem to rely on the gulping of air at the water surface for the initial activation of the swimbladder (Chatain, 1986; Kitajima et al., 1994; Bailey and Doroshov, 1995). Although the mechanisms and conditions for functional swimbladder inflation achievement vary among fish, its initial inflation seems to take place during a particular and finite interval, generally associated to the critical time of transition from endogenous to exogenous feeding (Trotter et al., 2005).

Correct swimbladder inflation is essential for functional buoyancy control, swimming ability and feeding success. Failure to inflate the swimbladder has been regarded as a major obstacle in the rearing of important commercial species such as striped bass, *Morone saxatilis* (Martin-Robichaud and Peterson, 1998), sea bream, *Sparus auratus*, and European sea bass (Chatain, 1994). Fish lacking a functional swimbladder have been reported to show higher mortality (Chatain, 1986, 1987; Chapman et al., 1988a; Chatain and Dewavrin, 1989; Trotter et al., 2003), increased metabolic rate (Marty et al., 1995), delayed growth (Battaglene and Talbot, 1992; Crespo et al., 2001;

Jacquemond, 2004) and skeletal deformities (Chatain, 1994; Kitajima et al., 1994; Divanach et al., 1997; Jacquemond, 2004; Trotter et al., 2001). The rate of swimbladder inflation in some physostome and transient physostome larvae has been significantly improved by use of surface cleaning devices favouring access to the air-water interface (Chatain and Ounais-Guschemann, 1990). However, since other factors like tank hydrodynamics, light intensity, salinity, and temperature may contribute to hamper or preclude swimbladder inflation in these fish, specific sets of environmental variables are often required (Divanach et al., 1996). On the whole, as fish with uninflated swimbladders are useless for commercial purposes, early methods for detecting and separating them from normal fish have been developed in important hatchery-reared species (Chapman et al., 1988b; Chatain and Corrao, 1992; Jacquemond, 2004). Phenomena of hyper-inflation or hypertrophy of the swimbladder during larval stages are little investigated despite being known to cause considerable losses under unfavourable culture conditions in some species (Bagarinao and Kungvankij, 1986; Planas and Cunha, 1999).

Although the biotic and abiotic mechanisms capable of influencing initial swimbladder inflation in fish may be quite numerous, the environmental/culture conditions are generally regarded as main contributory factors (Zilberg et al., 2004). In contrast, little attention has been devoted so far to see whether the process of swimbladder inflation has a genetic basis for its expression too (Harrell et al., 2002; Zilberg et al., 2004).

The present work was undertaken in order to investigate possible parental effects on swimbladder inflation anomalies (non-inflation and hyper-inflation) observed in hatchery-reared sea bass larvae. For this purpose, we performed a genotype and pedigree analysis of sibling families originating from a full factorial crossing and maintained under communal rearing conditions.

2. Material and methods

2.1. Fish

Fish originated from a commercial sea bass broodstock held under natural conditions of photoperiod and temperature and spawned following a previously published protocol (Peruzzi and Chatain, 2000). A total of *n*=10 mature females received a single injection of Luteinizing Hormone Releasing Hormone (LHRHa) at 10 µg kg^{-1} body weight. Mature oocytes were obtained from 8 out of 10 females approximately 72 hours following hormonal injection. Eggs of individual dams were equally divided into 6 aliquots of 50 ml and each aliquot was fertilized with 0.5 ml of sperm drawn from a single male (*n*=6) according to a full-factorial mating design producing 48 full-sibling families (8 dams x 6 sires). Individual families were maintained in 12l cylindro-conical incubators placed in a thermo-regulated seawater system at 13°C (Saillant et al., 2001). Floating (alive) and sinking (dead) eggs were separated at embryonation (48 hours post-fertilization) by increasing the salinity to 40‰ and their total volume and estimated number measured following the method described by Chatain (1994). Only 24 families (4 dams x 6 sires) generated enough living eggs for the requirements of the experiment. Equal aliquots of embryonated eggs (5ml or approx. 5000 eggs) were sampled from these families, pooled, transferred into a 500l tank maintained at 13-14°C until 20 days post-hatching (dph) and then at 20°C following standard rearing procedures for sea bass (Peruzzi et al., 2004). Water quality was monitored by a daily control of temperature and salinity, and weekly check of oxygen level, pH, NH_3, NH_4^+, NO_2^-, and NO_3^- concentrations.

2.2. Sampling

Measurement of larvae (L_T, mm) and verification of swimbladder condition were performed using a profile projector (Nikon V12), while photographs were taken using a Zeiss microscope fitted with a video camera module (Visilog 5.2 ©Noesis Vision, Canada). Larvae with hyperinflated swimbladder were collected (*n*=100) from the surface of the tank between 15 and 25 dph. Larvae with normal and without functional swimbladder were sorted following the method described by

Chatain and Corrao (1992) at the end of the larval period (45 dph). They were counted by a photographical method (Chatain et al., 1996), sampled (n=100/group) and finally preserved in 95% ethyl alcohol for further genotyping.

2.3. Molecular analyses and parental assignments

Nuclear DNA was extracted by alkaline lysis from portions of ethanol preserved larvae (Saillant et al., 2002). Briefly, alcohol was allowed to evaporate at ambient temperature in an Eppendorf tube and the dry tissue lysed in NaOH 200 mM (3 hr, 55°C). The solution was then neutralized with tris-HCl 200 mM and pH adjusted to 8.

The primers of five polymorphic microsatellite loci, *Labrax-3*, *Labrax-13*, *Labrax-17*, *Labrax-29* (Garcia de Leon et al., 1995) and *Dla-22F* (Ciftci et al., 2002) were amplified by PCR. The general PCR protocol was: 50-100 ng DNA, 0.1-1.0 µM primer, 400 µM dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 U taq polymerase (AB gene). The PCR reactions were carried out on a GeneAmp 2700 thermal cycler (Applied Biosystems) using the following profile: 94°C for 10 min, followed by 37 cycles of 94°C for 20 s, 59°C for 30 s and 72°C for 60 s, with a final extension of 72°C for 10 min. Forward primers were labelled with fluorescent dyes. The PCR products were separated by electrophoresis using an ABIPrism® 3100 Genetic Analyzer for fluorescent-labelled products (Applied Biosystems). Alleles were scored using a GeneMapper® Software v3.7 package (Applied Biosystems).

2.4. Statistical analysis

Based on the microsatellite genotypes from the parents and offspring potential parent pairs were explored among the offspring by means of likelihood-based parental allocation using the software PAPA 1.1 (Duchesne et al., 2002). The allocation method implemented in this software is based on breeding likelihood (Sancristobal and Chevalet, 1997). Given an offspring genotype, the likelihood

of a parental pair of genotypes is defined as the probability of this pair breeding the offspring genotype among all of its possible descents.

Data concerning the swimbladder status were analyzed by contingency table analysis using χ^2 (Dagnelie, 1975). For normally-inflated fish (S^+), the observed (O) frequencies were compared to expected (E) equal proportions of individuals in each family. For non-inflated (S^-) and hyper-inflated groups (S^{++}), the observed frequencies were compared to expected frequencies weighted for the survival frequencies observed in normal fish (S^+) and calculated as follows:

$$E_i = O_{S^+i} N$$

with E_i being the expected frequency for the cell i^{th} within a group, N the total observations in that group, and O_{S^+i} the corresponding observed frequency in the S^+_i group.

Statistical analyses were performed using Statview™ SE+ software. Differences to the equilibrium were accepted as significant when $P<0.05$. All means were expressed as values ± 95% confidence interval (CI).

3. Results

Survival rate from the stage of embryonated eggs to 45 dph was 13%. The percentage of larvae affected by hyperinflation could not be exactly estimated but was around 1% of the total fish. These larvae were recorded in a period between 15 dph and 25 dph, and averaged 5.92 ± 0.17 mm to 10.95 ± 0.46 mm L_T. The proportion of larvae with normally inflated and non-inflated swimbladder recorded at 45 dph was 97% and 3% respectively. At this stage, the mean length of the larvae was approximately 15.80 ± 0.32 mm L_T. Examples of the three swimbladder conditions are illustrated in Fig.1.

One of the microsatellite loci, *Labrax-13*, was difficult to amplify in the multiplex system and was excluded from the study. The remaining four loci allowed the unambiguous assignment of 264 out of the 273 genotyped offspring (97 %) to a single parental pair. The representation of the

153 offspring in the different families and swimbladder conditions is given in Table 1., and the observed
154 and expected frequencies for each class are reported in Fig. 2. In both the normal and hyperinflated
155 group 23 of the 24 possible families were represented, whereas in the group without swimbladder
156 only 22 families were found.

157 In larvae with normal swimbladder, the observed frequencies significantly differed from an
158 expected random distribution, indicating differential survival both among half-sibling sire (χ^2 =
159 23.054; $P < 0.001$; $df = 5$) and dam ($\chi^2 = 9$; $P = 0.0292$; $df = 3$) families. In particular, survival of
160 offspring generated from sire 5 and dam 2 was three-fold superior ($\chi^2 = 6.37$; $P = 0.0116$; $df = 1$)
161 and two-fold superior ($\chi^2 = 4.35$; $P = 0.0370$; $df = 1$) to the other parents in the corresponding class,
162 respectively. After correction for the survival frequencies observed in normal fish, larvae with non-
163 inflated swimbladder showed significant differences in family size only among half-sibling sire
164 families ($\chi^2 = 38.557$; $P < 0.0001$; $df = 5$). Here, the number of larvae generated from sire 1, 2 and 6
165 were two-fold superior to those of the remaining sires ($\chi^2 = 188$; $P < 0.001$; $df = 1$). In larvae with
166 hyper-inflated swimbladder, imbalance in family size was found both among half-sibling sire (χ^2 =
167 37.082; $P < 0.0001$; $df = 5$) and dam ($\chi^2 = 24.21$; $P < 0.0001$; $df = 3$) families. Again, larvae from
168 sire 1, 2 and 6 accounted for more than twice those generated by the remaining male parents (χ^2 =
169 32; $P < 0.001$; $df = 1$), whereas dam 1 and 4 produced 1.6-fold more larvae than the other two
170 females ($\chi^2 = 23.85$; $P < 0.001$; $df = 1$).
171
172
173 **4. Discussion**

174 In cultured fish considerable variation exists in the ability of larvae to achieve correct
175 swimbladder inflation, and some species require adapted culture techniques. In the European sea
176 bass, initial swimbladder inflation is size-mediated and usually occurs around the time of transition
177 from yolk sac depletion to exogenous feeding, i.e. when the larvae measure on average 5.5 to 6.5
178 mm L_T (Chatain, 1986). In this work, communally reared sibling families developing a normal

swimbladder showed unequal survival at the end of the larval period, with the offspring generated from the best performing dam and sire each accounting for nearly 37% of the total. Imbalance in family size due to differential survival has been observed in this species by Garcia de Leon et al. (1998) using similar rearing conditions. These authors analyzed the performance of 9 sibling families in order to detect possible parental effects on various larval traits, including survival at 40dph, and reported up to two-fold variations in survival rates as a result of individual dam and sire effects. Parental influences on early survival of mass reared sea bass larvae were also observed by Saillant et al. (2001) using a larger crossing design. In their work, most of the parental effects on early survival were largely due to females or by the interaction between these and one particular male parent. This is also in agreement with our findings, where approximately 17% of the larvae with normally inflated swimbladder were siblings of the best performing female and male parent. Hence, all these results show that genetic components may be involved in the survival performance of sea bass larvae reared under communal conditions and that parental contributions are not simply additive but possibly interactive.

Failure to inflate the swimbladder is a major obstacle in hatchery-reared fish, and is generally regarded to result from the application of unsuitable culture practices though it has been reported occasionally in wild populations too (Egloff, 1996; Czesny et al., 2005). In the present work, sea bass larvae lacking functional swimbladders accounted for 3% of the total population at the end of the experimental phase (40 dph). Slightly higher rates (11%) of larvae displaying non-inflated swimbladders at the same age have been reported by other authors (De León et al., 1998; Saillant et al., 2002). Our results highlighted a significant imbalance in family size due to paternal effects after correction for the survival frequencies observed in normally developed larvae. This is not in agreement with De León et al. (1998) who reported no significant parental effect for such an anomaly using a lower number of families but comparable rearing techniques. In a different genetic approach, Zilberg et al. (2004) found some alterations in transcription of genes involved in cardiovascular or muscular functions and associated with the state of swimbladder non-inflation in

angel fish, *Pterophyllum scalare* (Cichlidae). These authors observed that this abnormal trait was accompanied by reduced expression of certain genes potentially causing the defect and increased transcription of others compensating for associated functional disorders. Even though the aetiology of swimbladder non-inflation was not clearly determined in angel fish, genomic alterations, environmental conditions or induced mutation were suggested as possible contributory factors.

Hyper-inflation of the swimbladder during larval stages is rarely cited despite causing considerable losses in some hatchery-reared species under improper culture conditions like gas hypersaturation and other stress-inducing factors, acting individually or in combination (Johnson and Katavic, 1984; Bagarinao and Kungvankij, 1986; Planas and Cunha, 1999). In cultured European sea bass, phenomena of hyper-inflation are largely controlled though occasional events are still observed in some experimental (Saillant et al., 2002) and commercial settings (Chatain and Peruzzi, pers. comm.). In all cases, the larvae show impeded swimming and feeding behaviour, float at the water surface and die of starvation within a few days. Our results would suggest that swimbladder hyper-inflation in sea bass, though predominantly influenced by environmental clues, might present a genetic basis for its expression too. In particular, this genetic component would involve both paternally and maternally inherited factors.

Overall, the results also suggest a possible correlation between the two anomalies regarding the sire effect, the same two male parents generating the bulk of larvae affected by non-inflation and hypertrophic conditions. Moreover, a better capacity to survive does not seem to correspond with an increased ability of achieving correct swimbladder inflation, as the best performing sire and dam do not appear to be those contributing less to both anomalies.

In this work, the relatively low number of larvae and families analyzed did not allow us to estimate full and half-sibling heritabilities of the observed swimbladder inflation conditions. Elsewhere, a study designed to estimate the heritability of the non-inflated swimbladder defect in striped bass, *M. saxatilis*, has shown a moderate genetic value (h^2=0.35) for full-sibling families, and a low value (h^2=0.04) for half-sib dam families (Harrell et al., 2002). As indicated by these

authors, the half-sibling heritabilities showed scarce additive genetic variance for improvement of this trait by selective breeding.

Although uninflated or hypertrophic swimbladders in sea bass are generally regarded to result from the application of unsuitable culture conditions, our findings support the hypothesis of some level of genetic influence associated with these defects too. If confirmed, this would point out an even more complex co-causative mechanism of abnormal swimbladder development in this species. Nevertheless, it is clear that the extent of genetic control over such traits can be further assessed only using a dataset involving a larger number of families and individuals.

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Legends

Table 1. Number of larvae with (a) normally inflated, (b) non-inflated, and (c) hyperinflated swimbladder assigned to the 24 full-sibling families using the microsatellite loci *Labrax-3*, *Labrax-13*, *Labrax-17*, and *Labrax-29*.

Fig.1. Photomicrographs of 20 dph (7-8 mm TL) sea bass larvae with (a) normal functional swimbladder, (b) hyper-inflated swimbladder, and (c) non-inflated swimbladder. Arrows indicate the location of swimbladders. Scale bars represent 1mm.

Fig.2. Observed (□) and expected (▩) numbers of larvae presenting normal (S^{+}), non-inflated (S^{-}) or hyper-inflated (S^{++}) swimbladders in paternal and maternal half-sibs. For non-inflated and hyper-inflated groups, the expected frequencies represent weighted values.

Table 1

Sire	Dam				
	1	2	3	4	***Total***
(a) normal					
1	3	3	2	2	10
2	2	2	3	4	11
3	2	6	3	1	12
4	1	5	3	0	9
5	5	14	4	7	30
6	1	2	5	3	11
Total	14	32	20	17	83
(b) non- inflated					
1	4	8	7	5	24
2	6	6	5	6	23
3	1	4	3	1	9
4	0	3	1	0	4
5	4	7	1	5	17
6	4	5	4	4	17
Total	19	33	21	21	94
(c) hyperinflated					
1	4	4	3	5	16
2	10	3	4	7	24
3	2	4	0	2	8
4	1	1	1	1	4
5	5	6	2	6	19
6	5	7	2	11	25
Total	27	25	12	32	96

Figure 1

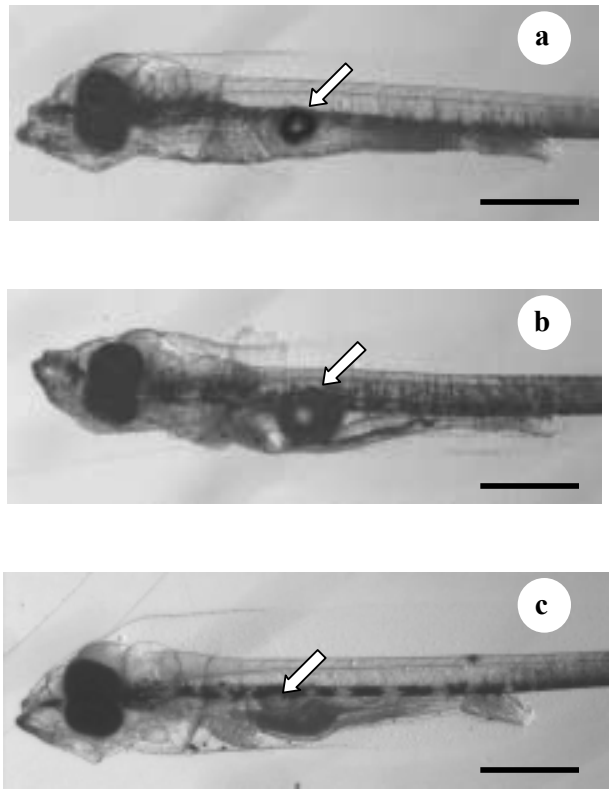
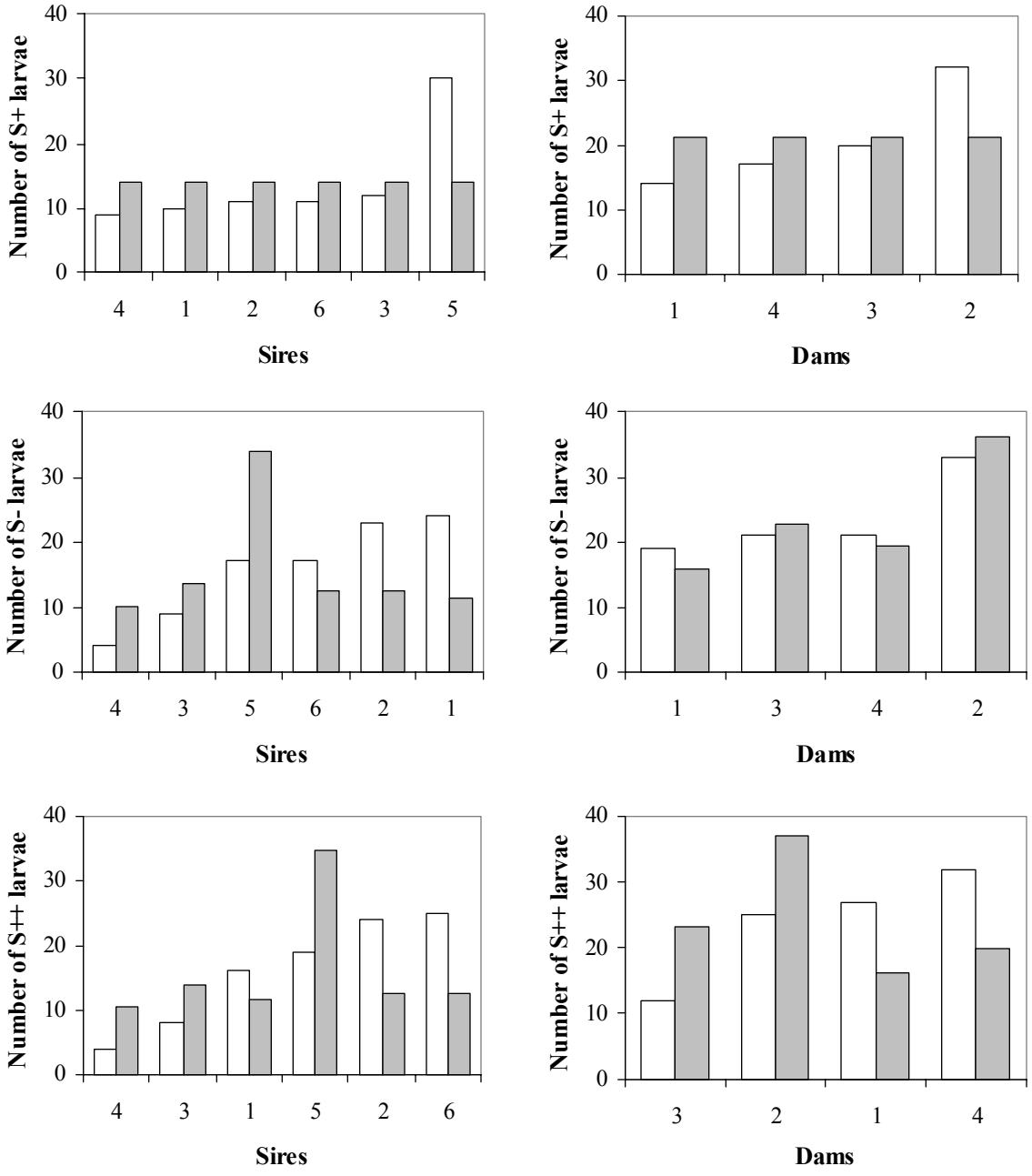


Figure 2


Number of S+ larvae
40
30
20
10
0
4 1 2 6 3 5
Sires
Number of S+ larvae
40
30
20
10
0
1 4 3 2
Dams
Number of S- larvae
40
30
20
10
0
4 3 5 6 2 1
Sires
Number of S- larvae
40
30
20
10
0
1 3 4 2
Dams
Number of S++ larvae
40
30
20
10
0
4 3 1 5 2 6
Sires
Number of S++ larvae
40
30
20
10
0
3 2 1 4
Dams