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The introduction of *Gambusia holbrooki* in rice field for mosquito control can positively affect rice production

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Abstract

The possibility to reduce the nitrogen input into rice cultivations thanks to the organic nitrogen input derived from the predator fish *Gambusia holbrooki* Girard used for the biological control of mosquito species was studied. Trials were conducted on three rice varieties, 'Koral', 'Gladio' and 'Loto', measuring the impact of different fish density and nitrogen inputs on phenological parameters (number of culms per square meter, culm height, spike length, number of fertile seeds per spike, and yield per hectare) and on the ammoniacal nitrogen concentration during the cultivation period. Trials were repeated for 2-3 years consecutively, and in all varieties we observed a positive effect of *G. holbrooki* on some phenological parameters (above all on the number of culms per square meter and on the number of seeds per spike) which in turn determined an increase of the yield per hectare, especially in those parcels with low nitrogen input. We hypothesize that the introduction of *G. holbrooki* in rice paddies exerted a positive role in maintaining the proper concentration of ammoniacal nitrogen thanks to the fish excreta, thus positively affecting rice cultivation during particularly sensitive phenological phases, like culm and spike formation. This practice could concur to reduce the need of nitrogen fertilization and, in turn, nitrogen pollution, while reducing mosquito larval density.

Key words: *Aedes* sp., *Culex* sp., nitrogen fertilization, mosquito control, mosquitofish, rice yield.

Introduction

Rice fields are one of the most important habitats for the pre-imaginal development of several mosquito species causing nuisance and/or sanitary concern (Lacey and Lacey, 1990). It is well known that in Italy the presence of extensive rice cultivations had a crucial role in the malaria epidemiology until pathogen eradication, which occurred after the Second World War (Raffaele, 1957). Today the main sanitary concern is caused by the role in vectoring arboviruses that some mosquito species can play. The most common mosquito species developing in Italian ricefields are: *Aedes caspius* (Pallas), *Anopheles atroparvus* van Thiel, *Anopheles labranchiae* Falleroni, *Anopheles maculipennis* Meigen, *Anopheles melanoon* Hackett, *Anopheles messeae* Falleroni, *Culex modestus* Ficalbi, *Culex pipiens* L.. Other species can be found with lower abundance, like *Aedes vexans* (Meigen), *Culex impudicus* Ficalbi, *Culex territans* Walker and *Uranotaenia unguiculata* Edwards.

Since 1996 the Piedmont Region has financially supported mosquito control programs with the Regional Law L.R. 75/1995. Due to their large extension in the region (approximately 200,000 hectares), rice cultivations appeared to be the most challenging situation.

We investigated the feasibility of an integrated mosquito control approach that combined the aerial distribution of *Bacillus thuringiensis israelensis* de Barjac (*Bti*) with the release of the eastern mosquitofish *Gambusia holbrooki* Girard, an efficient predator of mosquito larvae. *Bti* was generally used for the control of *Ae. caspius* developing during the first part of the rice cultivation cycle, when the paddies undergo a series of submersions and draught for agronomic reasons, while *G. holbrooki* was used in the second part of the cultivation cycle, when the submersion become permanent.

G. holbrooki and the sister species *Gambusia affinis* (Baird et Girard) are Poecilidae fishes, native of North America, highly tolerant in terms of water quality. Several studies have been conducted on the efficacy of the introduction of *Gambusia* for mosquito larvae control in different habitats, both natural and semi-natural (Maruashvili, 1990; Sato, 1989; Malhotra and Prakash, 1992), artificial ones (Castleberry and Cech, 1990; Mulligan *et al*., 1983; Offil and Walton, 1999) as well as in rice fields (Meisch, 1985). A number of factors can affect the efficacy of the method, and while Kottelat and Whitten (1996) in their review found from rare to non-existing effects on mosquitoes, most of the trials demonstrated the positive role of *Gambusia* in reducing/suppressing the larval mosquito populations (Meisch, 1985; Swanson *et al*., 1996).

Introduced to many countries for *Anopheles*/malaria control, today its release in new geographic areas arouses some concern because of the possible negative impact on the aquatic ecosystems (New South Wales National Parks and Wildlife Service, 2003). Particular concern regards its effect on the amphibian community and on mosquito predator invertebrates (New South Wales National Parks and Wildlife Service, 2003). Negative to perhaps neutral impact on native fishes by *G. affinis* were found by Kottelat and Whitten (1996). According to Miura *et al*. (1984), *G. affinis* at the rate of 224 fishes per hectare affected the population densities of cladocerans, mayflies, damselflies, notonectids and chironomids, while no impact was found on copepods, ostracods, corixids, dragonflies, belostomatids and aquatic beetles. The results of a study by Mischke *et al*. (2013) performed in catfish production ponds found no difference between ponds with or without mosquitofish in numbers and sizes of calanoid copepods, cyclopoid copepods, total copepods, *Bosmina* sp., *Ceriodaphnia* sp., *Moina* sp., *Daphnia* sp., or total cladocerans. Also water quality variables (soluble reactive phosphorus, nitrate, nitrite, ammonia, total nitrogen, total phosphorus, pH), phytoplankton density were similar between ponds with and without mosquitofish.

Akhurst *et al.* (2012) evaluate the effects of piscivorous Australian bass [*Macquaria novemaculeata* (Steindachner)], planktivorous gambusia (*G. holbrooki*), and benthivorous carp (*Cyprinus carpio* L.) on water quality in Emigrant Creek Dam, Australia, in 20 experimental enclosures (depth 1 m, volume 3.2 m^3), stocked at a density of 1875 kg/ha. Water turbidity, total suspended solids (TSS), total nitrogen (TN) and total phosphorus (TP) concentrations, and chlorophyll *a* (Chl-*a*), were all significantly higher in gambusia in comparison with the untreated enclosures. If these findings can be considered as a negative impact if the aim is to maintain high water quality in subtropical systems, in the case of rice production systems the introduction of *G. holbrooki* could be an alternative to nitrogen fertilization, with the additional, non-secondary effect of keeping mosquito population under control.

G. holbrooki can adapt very easily to the ricefield habitat, and since the 1970s integrated mosquito control programs including *G. holbrooki* have been conducted in California ricefields (Hoy and Reed, 1970; 1971; Hoy *et al.*, 1971; Kramer *et al.*, 1987; 1988), in Arkansas (Davey and Meisch, 1977), in Uzbekistan (Zainiev and Muminov, 1983) and in Italy (Bellini *et al.*, 1994).

While the role of *G. holbrooki* in mosquito control is therefore well documented, its impact on rice production has not been adequately investigated. From an agronomic point of view, the application of nitrogen fertilizers (N) to the soil in rice cultivation is a critical element to promote growth and tillering during the vegetative stage and to enhance spikelet production during the early panicle formation stage. If properly applied, N also contributes to grain filling by improving the photosynthetic capacity and enhancing carbohydrate accumulation in culms and leaf sheaths (Mae, 1997). The protein content of the seeds is known to be highly dependent on N availability, as their suitability to the industrial transformation is, too. While N has been recommended as a core fertilizer for rice in order to improve yields, an excessive N fertilization increases the risk of floral sterility and the sensitivity to pathogens and arthropod pests (Tanaka, 2010). Moreover, the outflow of excessive N from agricultural systems has been recognized as an international problem for the last 40 years (Commoner, 1975; Robertson and Vitousek, 2009).

The N input administrated by means of organic or mineral fertilization is transformed into ammoniacal N and nitric N. Part of the N input remains in the soil, while a variable portion is lost due to the leach, percolation and denitrification processes. During the different phenological phases, the rice plant roots receive the oxygen almost exclusively through the leaves. The rice plant's apical roots excrete the oxygen into the soil, thus favouring the oxidation of different compounds. Among them, the ammoniacal N is oxidated to nitrate, which is immediately adsorbed by the roots and translocated to the epigeal portions of the plant. Most of the ammoniacal N accumulates into the soil and as fast as it is consumed by oxidation it is replaced by the portion of ammoniacal N that is in solution in the water. In addition, the rice plant can also take the ammoniacal nitrose directly from the water, through the leaf sheath at the base of the culm and from the roots (Tinarelli, 1986).

The quantity of ammoniacal N produced by *G. holbrooki* plays an important role in this cycle. The aim of the present study was to evaluate the contribution of *G. holbrooki* excreta to the amount of the ammoniacal N in the water and its consequence on the rice cultivations parameters.

Materials and methods

Experimental design

The experimental design is summarized in table 1. In 1999, a preliminary study using the variety 'Koral', without N fertilization and applying a single density of *G. holbrooki*, was conducted in three large parcels (12,000 m^2). In the following three years, trials were planned to compare the combined effect of three different N fertilization levels and three *G. holbrooki* densities in four parcels of about 300 m^2 of the varieties 'Gladio' (2000-2002) and 'Loto' (2001-2002) (table 1).

The N fertilization was supplied in three fractions: before the sowing, at the shooting out and at flowering by using urea. The phosphate fertilization was not applied because the soil was known to be rich enough in phosphor, while the potassic fertilization was supplied at a dose of 150 kg/ha for all the treatments.

All varieties used in the experiments were sowed on dry soil. The release of *G. holbrooki* occurred 40 days after rice sowing, contemporarily with the permanent submersion of the rice parcels, according to a randomized block design. The fishes were collected from natural water bodies in Central Italy. Mixed batches (juvenile, adult males and females) were introduced in each parcel at the doses of 0 (Gh_0), 1,500 ($Gh_{1,500}$), 2,000 ($Gh_{2,000}$), 3,000 ($Gh_{3,000}$) and 4,000 ($Gh_{4,000}$) individuals/ha.

Table 1. Experimental design.

Year	Rice variety	Nitrogen fertilization levels (kg/ha)	*G. holbrooki* density (individuals/ha)	No. treatments/ var.	No. rep./ treatment	Total No. parcels	Parcel surface (m^2)
1999	Koral	0	0 - 1,500	2	3	6	12,000
2000	Gladio	100 -130 -160	0 - 2,000 - 4,000	9	4	36	330
2001	Gladio Loto	90 - 110 -130	0 - 1,500 - 3,000	9	4	72	375
2002	Gladio Loto	90 - 110 -130	0 - 1,500 - 3,000	9	4	72	286

Data collection

The data concerning the phenological parameters (number of fertile culms per square meter, culm height, spike length, number of fertile seeds per culm) were measured on 20 plants per parcel for each treatment. For the calculation of the yield per hectare at the optimal humidity level of 14% RH, recommended for the industrial processing, from the whole rice harvest of each parcel, 1 kg of rice per parcel was exsiccated at 105 °C for 48 h by means of an electric oven. The relative humidity was then measured on one sample of 50 g of rice for each parcel. Using these data, the weight of the rice produced in each parcel at the 14% RH was extrapolated and the total yield per hectare was calculated.

In the years 1999-2002, the concentration of the ammoniacal N in the water was detected. Seven to eight samplings were performed from June to August during the cultivation period of the varieties ‘Koral’ (1999) and ‘Gladio’ (2000-2002). During each sampling, four samples were collected in different parts of the parcel, using a 0.25 L capacity container. For each sample, the container was filled up by collecting the water from three points within one square meter. The container was hermetically closed, brought to the lab and frozen till the moment of the analyses.

Statistics and data analysis

In 1999, for the variety ‘Koral’, the effect of *G. holbrooki* on the number of culms per square meter, culm height, spike length, number of seeds per spike and total yield per hectare was studied by means of one-way ANOVA and the Tukey test was used for means’ separation.

In 2000-2002, for the varieties ‘Gladio’ and ‘Loto’, separately for each year, two-way ANOVA tests were run in order to evaluate the effect of the presence of *G. holbrooki* together with different levels of N fertilization on the above mentioned phenological parameters and on the total yield per hectare. The Tukey test was applied for means’ separation.

For each study year, the results of the laboratory analyses on the ammoniacal N concentration in the water samples were statistically analyzed by means of two-way ANOVA, considering the *G. holbrooki* density and the period (i.e. the sampling date) as main effects.

Results

Variety ‘Koral’ 1999

The introduction of *G. holbrooki* significantly increased the number of culms per square meter from 299.3 ± 7.6 to 317.3 ± 6.1 (table 2) while it had no statistically significant influence on culm height (Gh_0 86.6 ± 3.4 mm; $Gh_{3,000}$ 92.3 ± 4.9 mm) (table 3) and spike length (Gh_0 18.6 ± 0.7 mm; $Gh_{3,000}$ 18.7 ± 0.4 mm) (table 4). A statistically significant increase of the yield per hectare in *G. holbrooki* parcels (71.6 q/ha) was observed with respect to those without fishes (68.5 q/ha) (table 5).

Variety ‘Gladio’ 2000-2002

Number of culms

In 2000, the number of culms per square meter was positively affected by the fertilization level (figure 1, table 2), with a statistically significant increase in the parcels with 110 and 130 kg/ha of N (N_{110} and N_{130} from now on) compared to those with 90 kg/ha of N (N_{90} from now on) (Tukey test, respectively: P = 0.0230 and P = 0.0005). In 2001, the highest fertilization levels produced a higher number of culms per square meter compared to the lowest (Tukey test: P = 0.0021); no statistically significant difference emerged from the other paired comparisons (Tukey test: all P > 0.05). In 2002, the presence of *G. holbrooki*, but not the N level, affected the number of culms per square meter (table 2) with higher numbers of culms in the parcels with 3,000 *G. holbrooki* individuals ($Gh_{3,000}$ from now on) with respect to $Gh_{1,500}$ or Gh_0. (Tukey test, respectively: P = 0.0024 and P = 0.0306). In none of the three trial years statistically significant interactions among the two main variables were found (table 2).

Table 2. ANOVA statistics for the number of culms per m^2 in the four years of trials.

Year	Variety	Source of variation	SS	DF	MS	F	P
1999	Koral	*G. holbrooki*	486	1	486	10.27	**0.0327**
2000	Gladio	*G. holbrooki*	772	2	386	1.17	0.3267
		N level	6,817	2	3,408	10.31	**0.0005**
		G. holbrooki × N level	1,322	4	330	1.00	0.4250
2001	Gladio	*G. holbrooki*	1,589	2	795	1.43	0.2564
		N level	8,177	2	4,089	7.37	**0.0028**
		G. holbrooki × N level	1,005	4	251	0.45	0.7693
2001	Loto	*G. holbrooki*	1,358	2	679	3.47	**0.0454**
		N level	8,253	2	4,126	21.10	**0.0000**
		G. holbrooki × N level	1,113	4	278	1.42	0.2530
2002	Gladio	*G. holbrooki*	7,671	2	3,835	7.52	**0.0025**
		N level	2,344	2	1,172	2.30	0.1198
		G. holbrooki × N level	1,338	4	334	0.66	0.6279
2002	Loto	*G. holbrooki*	2,041	2	1,020	10.02	**0.0005**
		N level	789	2	395	3.88	**0.0331**
		G. holbrooki × N level	743	4	186	1.82	0.1533

Table 3. ANOVA statistics for the culm height in the four years of trials.

Year	Variety	Source of variation	SS	DF	MS	F	P
1999	Koral	*G. holbrooki*	49.31	1	49.31	2.778	0.1709
2000	Gladio	*G. holbrooki*	28.31	2	14.0	1.87	0.1728
		N level	35.01	2	17.5	2.34	0.1156
		G. holbrooki × N level	7.12	4	1.81	0.24	0.9156
2001	Gladio	*G. holbrooki*	5.17	2	2.58	1.62	0.2161
		N level	4.17	2	2.08	1.31	0.2869
		G. holbrooki × N level	0.67	4	0.17	0.10	0.9799
2001	Loto	*G. holbrooki*	18.7	2	9.4	2.30	0.1145
		N level	13.7	2	6.9	1.69	0.1663
		G. holbrooki × N level	16.8	4	4.2	1.03	0.8639
2002	Gladio	*G. holbrooki*	13.50	2	6.75	3.23	0.0554
		N level	10.17	2	5.08	2.43	0.1071
		G. holbrooki × N level	6.83	4	1.71	0.82	0.5259
2002	Loto	*G. holbrooki*	0.451	2	0.225	2.08	0.1192
		N level	0.284	2	0.142	1.31	0.2038
		G. holbrooki × N level	0.653	4	0.163	1.51	0.4089

Table 4. ANOVA statistics for the spike length in the four years of trial.

Year	Variety	Source of variation	SS	DF	MS	F	P
1999	Koral	*G. holbrooki*	0.027	1	0.027	0.078	0.7938
2000	Gladio	*G. holbrooki*	1.047	2	0.523	5.7	**0.0087**
		N level	4.807	2	2.403	26.1	**0.0000**
		G. holbrooki × N level	0.402	4	0.100	1.1	0.3808
2001	Gladio	*G. holbrooki*	1,500	2	0.750	0.711	0.5003
		N level	6,500	2	3.250	3.079	0.0624
		G. holbrooki × N level	0.500	4	0.125	0.118	0.9748
2001	Loto	*G. holbrooki*	1.056	2	0.528	1.12	0.3417
		N level	2.056	2	1.028	2.18	0.1329
		G. holbrooki × N level	0.444	4	0.111	0.24	0.9159
2002	Gladio	*G. holbrooki*	0.077	2	0.039	0.12	0.8901
		N level	2.024	2	1.012	3.06	0.0632
		G. holbrooki × N level	2.496	4	0.624	1.89	0.1411
2002	Loto	*G. holbrooki*	0.451	2	0.225	2.08	0.1443
		N level	0.284	2	0.142	1.31	0.2860
		G. holbrooki × N level	0.653	4	0.163	1.51	0.2278

Table 5. ANOVA statistics for the rice yield (q/ha) in the four years of trials.

Year	Variety	Source of variation	SS	DF	MS	F	P
1999	Koral	*G. holbrooki*	46.48	1	46.48	27.69	**0.0001**
2000	Gladio	*G. holbrooki*	248.20	2	124.10	8.81	**0.0011**
		N level	980.00	2	490.00	34.79	**0.0000**
		G. holbrooki × N level	10.70	4	2.70	0.19	0.9416
2001	Gladio	*G. holbrooki*	64.34	2	32.17	4.44	**0.0215**
		N level	368.08	2	184.04	25.40	**0.0000**
		G. holbrooki × N level	12.31	4	3.08	0.42	0.7895
2001	Loto	*G. holbrooki*	11.55	2	5.77	0.242	**0.0032**
		N level	332.76	2	166.38	6.975	**0.0000**
		G. holbrooki × N level	24.28	4	6.07	0.254	0.7646
2002	Gladio	*G. holbrooki*	97.81	2	48.91	4.07	**0.0285**
		N level	747.07	2	373.53	31.09	**0.0000**
		G. holbrooki × N level	9.53	4	2.38	0.20	0.9370
2002	Loto	*G. holbrooki*	130.3	2	65.1	3.159	0.0585
		N level	319.6	2	159.8	7.748	**0.0021**
		G. holbrooki × N level	9.2	4	2.3	0.111	0.9775

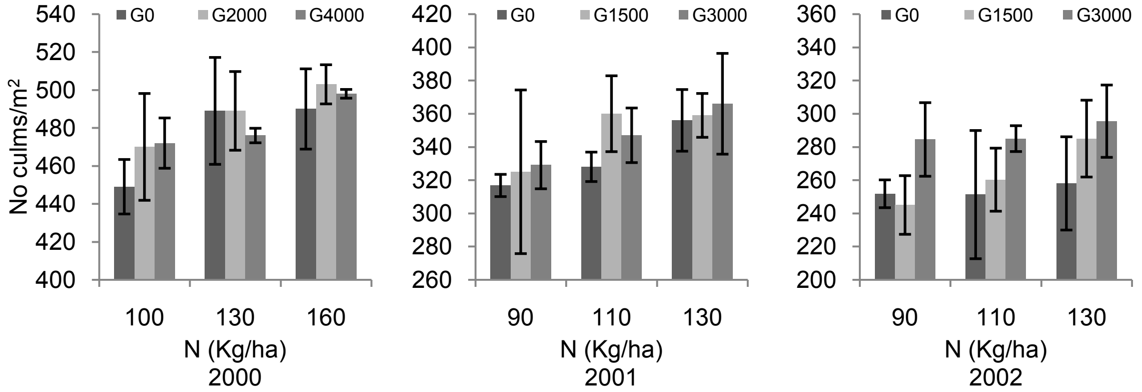

Figure 1. Number of culms per square meter of the var. 'Gladio' observed in the three years of trials for the different treatments.

Culm height

Culm height was not significantly affected either by *G. holbrooki* presence or N level in any of the years of study (figure 2, table 3).

Spike length

Spike length was significantly affected by N fertilization and by *G. holbrooki* doses in 2000 (figure 3, table 4). A significant increase was observed in the N_{130} parcels in comparison to the N_{110} and N_{90} ones, and also between the latter two (Tukey test, respectively: P = 0.001, P = 0.0004, P = 0.0424), as well as in the $Gh_{2,000}$ parcels compared to the Gh_0 ones. While in 2001 and 2002 no significant increase in spike length was observed in the parcels treated with the medium and high N doses (table 4) as well as in the parcels with the highest *Gambusia* density ($Gh_{4,000}$) (Tukey test: Gh_0 vs $Gh_{2,000}$ P = 0.0064; Gh_0 vs $Gh_{4,000}$ P = 0.3161; $Gh_{2,000}$ vs $Gh_{4,000}$ P = 0.1628) (table 4).

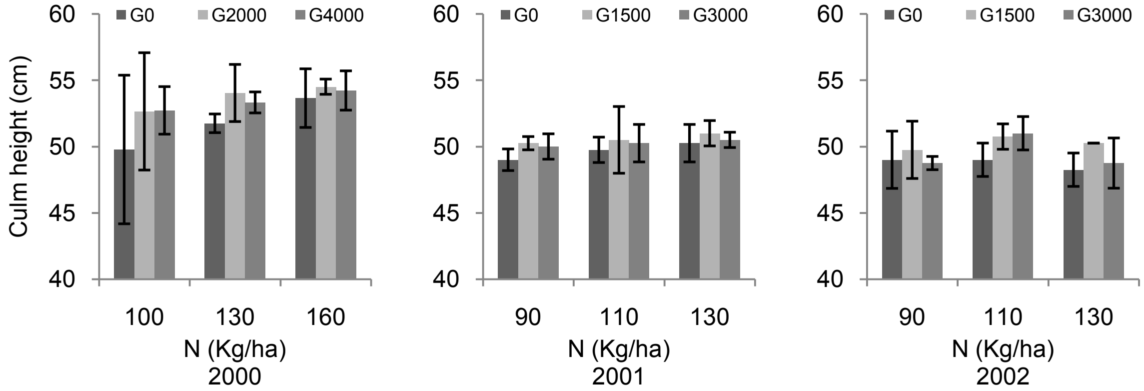

Figure 2. Culm height of the var. 'Gladio' observed in the three years of trials for the different treatments.

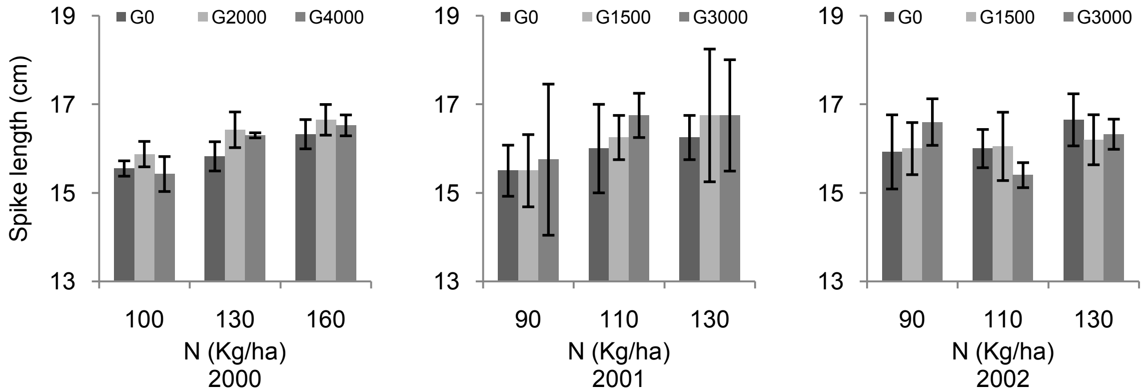

Figure 3. Spike length of the var. 'Gladio' observed in the three years of trials for the different treatments.

Number of fertile seeds

The number of fertile seeds per culm was positively affected both by N fertilization and *G. holbrooki* in 2000, with no statistically significant interaction between the two factors (figure 4, table 6). The number of fertile seeds per culm was statistically higher in the N_{130} and N_{160} parcels than the N_{100} ones (Tukey test: N_{100} vs N_{130} P = 0.0006; N_{100} vs N_{160} P = 0.0001; N_{130} vs N_{160} P = 0.1686) and in the $Gh_{4,000}$ and $Gh_{2,000}$ parcels compared to the control parcels without fishes (Tukey test: Gh_0 vs $Gh_{2,000}$ P = 0.0045; Gh_0 vs $Gh_{4,000}$ P = 0.0094; $Gh_{2,000}$ vs $Gh_{4,000}$ P = 0.9522). In the 2001 and 2002, no statistically significant difference in the number of fertile seeds per culm in the parcels with different treatments was found.

Rice productivity

The total yield per hectare was positively affected both by N fertilization levels and by *G. holbrooki*, in all trial years (figure 5, table 5). In 2000, a higher yield was obtained in the $Gh_{2,000}$ and $Gh_{4,000}$ parcels compared to the Gh_0 ones (Tukey test: Gh_0 vs $Gh_{2,000}$ P = 0.003; Gh_0 vs $Gh_{4,000}$ P = 0.004; $Gh_{2,000}$ vs $Gh_{4,000}$ P = 0.98). In 2001 and 2002, only the highest *G. holbrooki* dose was effective in increasing the total yield per hectare (Tukey test, 2001: Gh_0 vs $Gh_{1,500}$ P = 0.053; Gh_0 vs $Gh_{3,000}$ P = 0.032; $Gh_{1,500}$ vs $Gh_{3,000}$ P = 0.97; 2002: Gh_0 vs $Gh_{1,500}$ P = 0.79; Gh_0 vs $Gh_{3,000}$ P = 0.029; $Gh_{1,500}$ vs $Gh_{3,000}$ P = 0.11).

Both the medium and high N dose increased the rice production in contrast to the lowest fertilization dose. Moreover the highest fertilization level evidenced the highest productivity.

Variety 'Loto' 2001-2002

Number of culms

The number of culms per square meter was positively affected by the two main factors in both the years of study (figure 6), without statistically significant interactions between them (table 2). In 2001, the paired comparisons of means showed statistically higher values for the parcels treated with the medium and high N levels (Tukey test, N_{90} vs N_{110}: P = 0.0018; N_{90} vs N_{130}: P = 0.0001; N_{110} vs N_{130}: P = 0.0084) and for the medium and high *G. holbrooki* densities in comparison to the parcels without fishes ($Gh_{1,500}$ vs Gh_0: P = 0.0373; $Gh_{3,000}$ vs Gh_0: P = 0.0245; $Gh_{3,000}$ vs $Gh_{1,500}$: P = 0.8622).

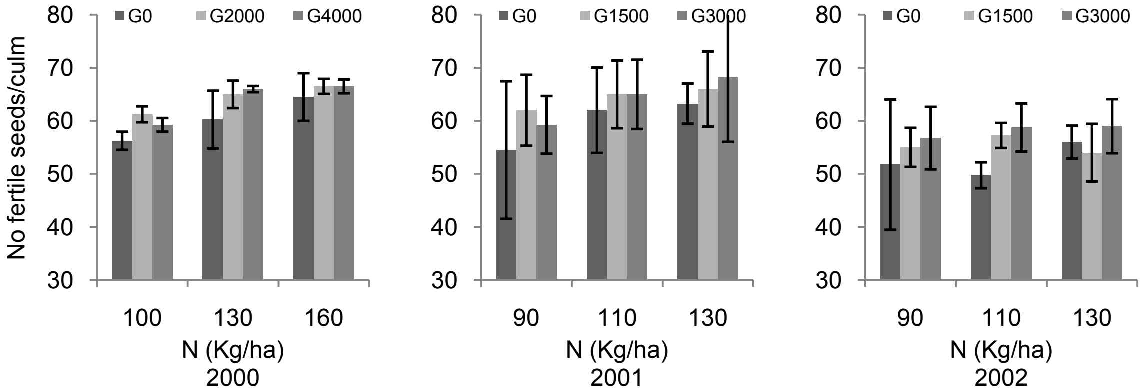

Figure 4. Number of fertile seeds per culm of the var. 'Gladio' observed in the three years of trials for the different treatments.

Table 6. ANOVA statistics for the number of fertile seeds per culm in the three years of trials.

Year	Variety	Source of variation	SS	DF	MS	F	P
2000	Gladio	*G. holbrooki*	113.2	2	56.6	7.56	**0.0246**
		N level	302.2	2	151.2	29.19	**0.0000**
		G. holbrooki × N level	23.7	4	5.9	0.79	0.5432
2001	Gladio	*G. holbrooki*	150.4	2	75.2	1.12	0.3407
		N level	341.2	2	170.5	2.54	0.0974
		G. holbrooki × N level	38.9	4	9.7	0.15	0.9636
2001	Loto	*G. holbrooki*	144.5	2	72.2	2.537	0.0978
		N level	326.2	2	163.1	5.726	**0.0084**
		G. holbrooki × N level	11.3	4	2.8	0.099	0.9817
2002	Gladio	*G. holbrooki*	192.7	2	96.4	2.92	0.0709
		N level	20.4	2	10.2	0.30	0.7365
		G. holbrooki × N level	95.4	4	23.9	0.72	0.5832
2002	Loto	*G. holbrooki*	59.7	2	29.9	1.847	0.0585
		N level	151.1	2	75.5	4.672	**0.0022**
		G. holbrooki × N level	79.3	4	19.8	1.226	0.9776

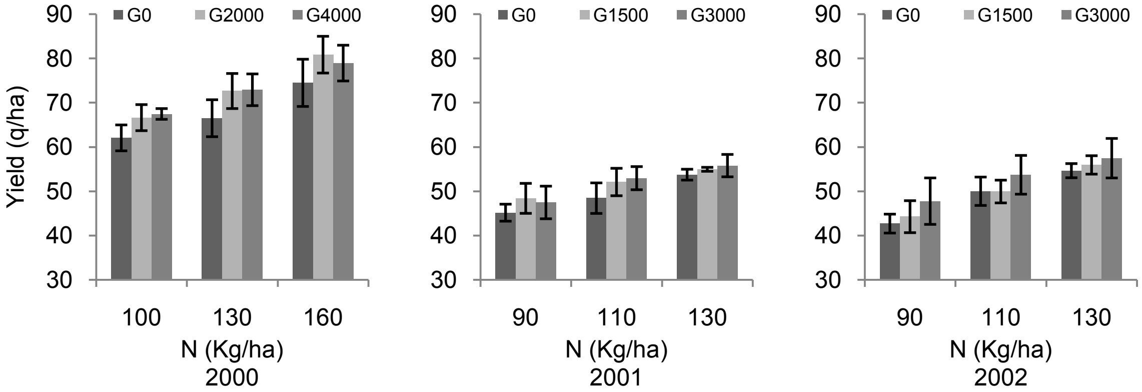

Figure 5. Total yield per hectare of the var. 'Gladio' observed in the three years of trials for the different treatments.

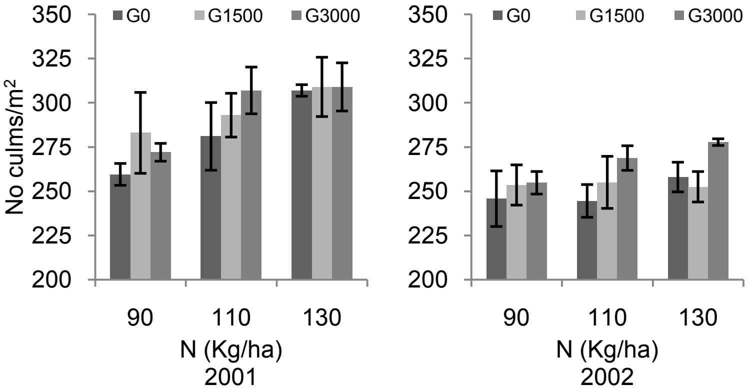

Figure 6. Number of culms per square meter of the var. 'Loto' observed in the two years of trials for the different treatments.

Culm height and spike length

Both parameters were not influenced by the two main factors in none of the study years (figures 7-8, tables 3-4).

Number of fertile seeds

The number of fertile seeds per culm was affected by the N fertilization level but not by *G. holbrooki* density, even if in 2000 the values registered for this parameter were on average higher for the *G. holbrooki* parcels with respect to the others (figure 9, table 6). The highest N fertilization rate produced a higher number of fertile seeds per culm than the lowest N rate. None of the other paired comparisons showed statistically significant differences (Tukey test, N_{90} vs N_{110} P = 0.0682; N_{90} vs N_{130} P = 0.0077; N_{110} vs N_{130} P = 0.6103).

Rice productivity

The rice yield per hectare was influenced by the N level both in 2001 and in 2002 (table 5 and figure 10). The presence of *G. holbrooki* affected the rice yield in 2001 and at a probability level slightly higher than 5% (P = 0.05831) in 2002 (table 5) and we found significant differences between the highest *G. holbrooki* density

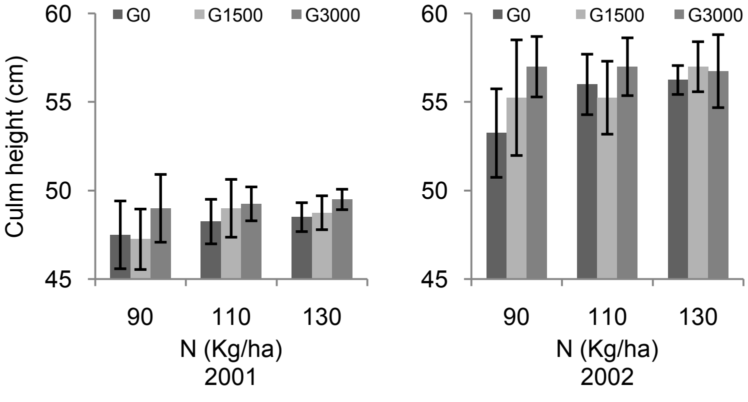

Figure 7. Culm height of the var. 'Loto' observed in the two years of trials for the different treatments.

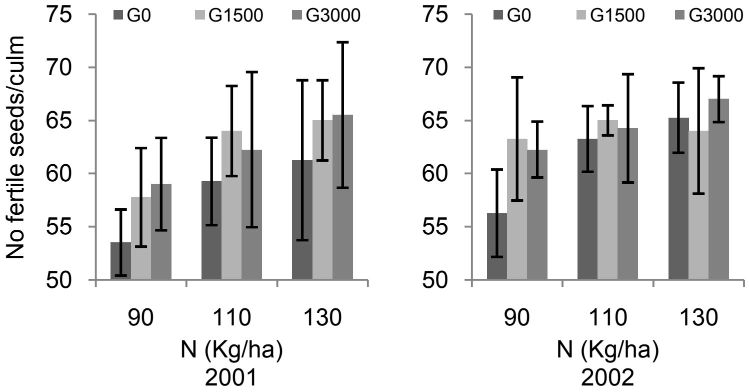

Figure 8. Number of fertile seeds per culm of the var. 'Loto' observed for the different treatments in the two years of trials.

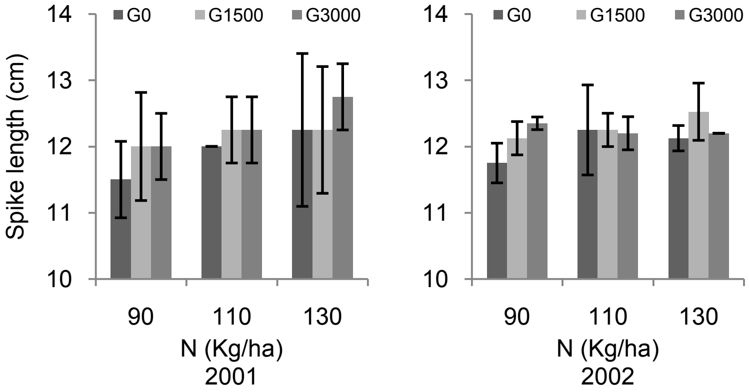

Figure 9. Spike length of the var. 'Loto' observed in the three years of trials for the different treatments.

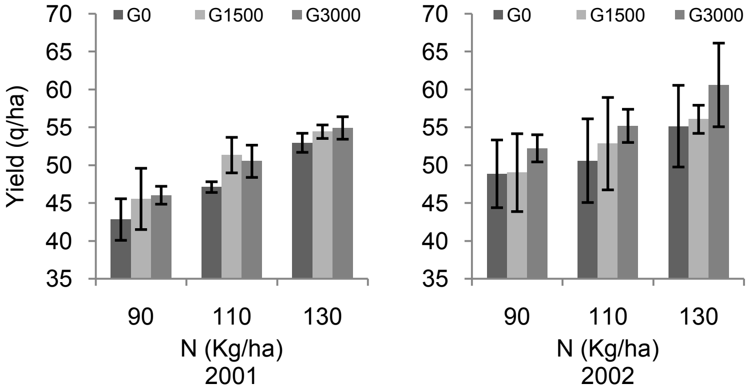

Figure 10. Total yield per hectare of the var. 'Loto' observed in the two years of trials for the different treatments.

and the non treated control in 2001 (Tukey test, 2001: Gh_0 vs $Gh_{1,500}$ $P = 0.009$; Gh_0 vs $Gh_{3,000}$ $P = 0.007$; $Gh_{1,500}$ vs $Gh_{3,000}$ $P = 0.99$), while no significant differences were observed in the 2002 (figure 10).

Ammoniacal nitrogen concentration

The chemical analyses of water in 2000, 2001 and 2002 showed an increase of the concentration of ammoniacal N in the *G. holbrooki* parcels in contrast to the non treated ones, but only in 2000 a statistically significant difference emerged between the $Gh_{2,000}$ parcels in comparison to the others (figure 11, table 7).

Discussion

In general, for all the tested rice varieties the presence of *G. holbrooki* positively affected at least one of the phenological parameters observed, and this resulted in an increase of the total yield in comparison with the control parcels without fishes, but often without statistically significant differences between the *G. holbrooki* densities tested.

Regarding the 'Koral' variety, *G. holbrooki* produced an increase of the number of culms per square meter which in turn determined an increase of the rice yield per ha.

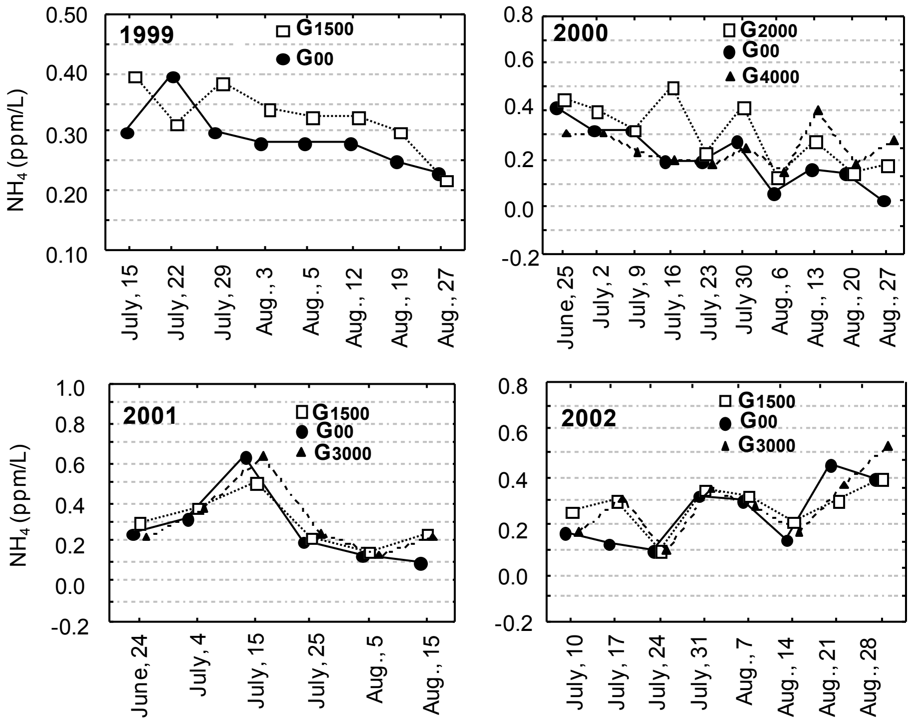

Figure 11. Ammoniacal nitrogen concentration dynamic in the water during the four years of trials.

Table 7. Statistics on the effect of *G. holbrooki* on NH_4 (ppm/l) concentration in the water.

Year	Variety	Source of variation	SS	DF	MS	F	P
		G. holbrooki	0.014	1	0.014	5.029	**0.0319**
1999	Koral	Sampling date	0.078	7	0.011	3.858	**0.0037**
		G. holbrooki × sampling date	0.035	7	0.005	1.740	0.1348
		G. holbrooki	0.166	2	0.083	6.8734	**0.0017**
2000	Gladio	Sampling date	0.913	9	0.101	8.3847	**0.0000**
		G. holbrooki × sampling date	0.488	18	0.027	2.2449	**0.0067**
		G. holbrooki	0.014	2	0.006	0.2511	0.7789
2001	Gladio	Sampling date	1.475	5	0.295	10.611	**0.0000**
		G. holbrooki × sampling date	0.102	10	0.010	0.3658	0.9600
		G. holbrooki	0.020	2	0.009	0.7037	0.4983
2002	Gladio	Sampling date	1.045	7	0.149	10.575	**0.0000**
		G. holbrooki × sampling date	0.185	14	0.013	0.9344	0.5277

In the ‘Gladio’ variety, N fertilization positively affected the number of fertile culms per square meter in 2000 and 2001, the number of fertile seeds per culm in 2000, and the spike length in all the three years of trials. The presence of *G. holbrooki* increased the number of fertile culms per square meter and the culm height in 2002, the number of fertile seeds per culm and the spike length in 2000. Both factors, in all the trial years, increased the total yield per hectare.

In the ‘Loto’ variety, in both study years, N fertilization affected the number of culms per square meter, the number of fertile seeds per culm and the rice yield per hectare. *G. holbrooki* increased the number of culms per square meter and the total yield in both study years. Thus, it can be stated that both main factors analyzed positively affected rice cultivation output of the three varieties under investigation for all the years of study, never showing statistically significant interactions. Our findings seem to indicate that *G. holbrooki* contributed to maintain available the appropriate amount of ammoniacal nitrogen in the water and that the continuity of N supply ensured by the fishes compensated possible insufficient N chemical input. As an example, in the variety ‘Loto’ in 2002, the parcels with *G. holbrooki* produced a higher number of culms per square meter in contrast to the control parcels without fishes. Moreover, comparing the parcels with different N input, the yield increase was minimized in the parcels that received the highest N dose, and maximized in the parcels without N fertilization but with the fishes. Analogous trends can be seen for the variety ‘Gladio’ in the three years of study. The excess of nitrogen fertilization could explain why in 2000, in the parcels with the highest *G. holbrooki* density ($Gh_{4,000}$) and the maximum N input, decreased total yield data were registered. In summary, the increased availability of ammoniacal N supplied by *G. holbrooki* excreta determined an increase in the number of culms per square meter and in the total yield, which was only slightly different from the yield obtained in the parcels with the highest N dose fertilization without fishes. The water chemical analyses in 2000, 2001 and 2002 showed an increase of the ammoniacal nitrogen concentration in *G. holbrooki* parcels compared to the untreated ones, but only in the years 1999-2000 statistically significant differences emerged (table 7).

Our conclusion is that the introduction of *G. holbrooki* into the rice paddies during the permanent submersion phase positively affect some phenological parameters (mainly the number of culms per square meter and the number of seeds per spike) and, in turn, the total yield per hectare. As the mere increase in N applications does not guarantee yield improvement, and a decrease in crop N use efficiency has been observed with increasing N input (Fageria and Baligar, 2001; Jiang *et al*., 2004; Peng *et al*., 2006), we hypothesise that the positive effect exerted by the fishes has to be related to the gradual and continuous release of ammoniacal nitrogen which constitutes an immediately available nitrogen source for the plant that can compensate the insufficient supply of nitrogen when (even provisionally) it becomes unavailable during the crucial flowering and spikelet phases. In addition, we estimated that the positive role played by the presence of the proper number of *G. holbrooki* in the rice paddies can allow reduction of 15-18% of the nitrogen fertilization input without reducing the total yield.

Our study demonstrated that an integrated mosquito control program in rice fields, that includes also the use of *G. holbrooki*, can result in a positive effect from the agronomic point of view, in agreement with a number of findings obtained all around the world concerning the fish-farming in rice fields (Fernando, 1993). Further investigations would be useful to better quantify the possible contribution of *G. holbrooki* to rice cultivation comparing different varieties and different soil composition and climate conditions.

Some concern remains about the possible negative effect of the predation activity of *G. holbrooki* towards invertebrate and vertebrate species, particularly amphibians, and of its contribution to eutrophication due to zooplankton removal and phytoplankton enhancement. Burgett *et al*. (2007) found that the presence of mosquitofish chemical cues affect wood frog (*Rana sylvatica* LeConte) tadpole behavior, but the ammonium nitrate (≥ 50 mg L^{-1} NH_4NO_3) directly decreased their survivorship. Cardona (2006) in enclosure experiments, confirmed the effect of zooplankton density decrease (more evident for cladocerans and ostracods than for cyclopoid copepods), whilst rotifer density was not modified. No differences were observed between the chlorophyll concentration in fish and fish-less enclosures. The only benthic macroinvertebrate species whose density increased in the absence of mosquitofish was the mud snail *Hydrobia acuta* (Draparnaud). The density of chironomid midge larvae and damselflies did not increase in fish-less enclosures. Nitrogen concentration decreased after fish exclusion, but phosphorus concentration remain unchanged. Cardona (2006) concluded that the eastern mosquitofish affect zooplankton of the Mediterranean oligohaline lagoons considerably, but they do not enhance phytoplankton growth, because the system is bottom-controlled by submerged macrophytes. In conclusion, we feel confident that further studies will demonstrate the global environmental advantage of the controlled use of *G. holbrooki* on a large scale in rice fields. Balancing between negative and positive effects of the introduction of *G. holbrooki* in rice paddies, it has to be considered that, in addition to nitrogen fertilization, in order to control mosquito populations the only alternative in many areas of the world is the use of large amounts of insecticides that produce water pollution and constitute a major hazard for many invertebrate and vertebrate species.

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