

## RESPONSES IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) TO INCREASING DIETARY DOSE OF LUPININE ALKALOID

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### ABSTRACT

**Yellow lupin (*Lupinus luteus*) is a promising source of protein in feeds for carnivorous fish. However, the high content of alkaloids may limit its potential for use. Lupinine is the main quinolizidine alkaloid in several varieties of *Lupinus luteus*. It has been reported to be highly toxic for bacteria and invertebrates, however no information is available about the allelopathic effects of lupinine on vertebrates. This study investigated the effect of increasing dietary doses of lupinine on feed intake, growth performance, tissue histology and nutritional composition of rainbow trout. Duplicate groups of rainbow trout (initial body weight of 0.3 kg) were fed extruded fish meal based diets containing 0, 50, 75, 100, 250, 500, 1000 and 5000 mg lupinine/kg for 60 days. Results from this study show that feed intake and growth were linearly reduced in response to dietary lupinine. Based on results obtained by additional analysis of variance, the practical tolerance levels of lupinine with regard to growth and feed intake was up to 100 mg/kg feed. Carcass composition did not vary among treatment. Besides a depletion of glycogen and lipid stores in the hepatocytes, the dietary inclusion of lupinine did not cause any morphological changes in kidney, heart or intestinal tissue. These results indicate that the lupinine alkaloid possesses a strong anti-palatability effect, but does not pose an adverse short-term risk to the health of rainbow trout.**

### KEYWORDS

lupinine, yellow lupin, anti-nutritional factors, fish nutrition

### INTRODUCTION

Alkaloids are secondary metabolites in plants; their main function is chemical defence against herbivores and microorganisms. They taste bitter and are toxic to vertebrates (including human beings) and insects. The toxic effect of alkaloids can be explained through their interaction with: acetylcholine receptors, Na<sup>+</sup> and K<sup>2</sup>

channels (Korper *et al.* 1998), DNA and RNA enzymes and protein biosynthesis (Wink *et al.* 1998). At the organ level, alkaloids may affect the brain, muscle, heart, lungs, kidneys, liver, bones and also related physiology such as digestion, respiration, blood circulation, hormonal regulation and reproduction (Wink, 1998).

These compounds are synthesised as quinolizidine, peridine and indole alkaloids by lupin plants, which is the only alkaloid-containing legume likely to be used as an ingredient in aquafeed (Tacon, 1997; Francis *et al.* 2001; Glencross *et al.* 2007).

Acute toxicity effects of lupin quinolizidine alkaloids have been reported in rodents (Robbins *et al.* 1996; Pothier *et al.* 1998), chickens (Oliver and Jonker, 1997), ducks (Oliver and Jonker, 1998) and pigs (Godfrey *et al.* 1995); whereas no information is available for any fish species. Among the domesticated lupin species, *Lupinus luteus* is a good prospect for aquafeeds (Glencross *et al.* 2002; Glencross *et al.* 2004), displaying significant nutritional advantages over others legumes, due to higher levels of protein and sulphur-containing amino acids (Sujak *et al.* 2006). However, inclusion of a high level of *Lupinus luteus* in aquafeed is limited by the alkaloid levels in the seed, which can be higher than those in other varieties of domesticated lupins (Pettersen *et al.* 1997). Low-alkaloid *Lupinus luteus* varieties have been produced by breeding programs, but these are agronomically costly to cultivate due to the need for high levels of insecticides as a means to confront insect infestation problems. Lupinine is the main quinolizidine alkaloid in some *Lupinus luteus* varieties (Wink *et al.* 1995). Significant bactericide properties have been reported of this alkaloid (de la Vega *et al.* 1996; Wink *et al.* 1998), nevertheless its allelopathic dose-response effects on vertebrates has not been documented. Considering, the high potential of *Lupinus luteus* as an ingredient in fish diets and the ecological importance of alkaloids for the fitness of lupins, it is important to know the effect of lupinine on fish, in order

to optimise the production and use of *Lupinus luteus* as a fish feed ingredient.

The aim of this study was to examine the influence of dietary inclusion of lupinine on growth performance, tissue histology and nutritional composition of rainbow trout (*Oncorhynchus mykiss*), and to determine its acute toxicity concentration.

## MATERIALS AND METHODS

### DIETS

The diets contained LT-fish meal as the main protein source and were formulated to contain 45% crude protein and 20% lipids (DM basis). The diets were extruded (Bühler twin-screw extruder; DNDG 62, Uzwil, Switzerland) and pelleted prior to the addition of lipids and lupinine. Lupinine powder (97% purity, Alfa Aesar GmbH & Co, Karlsruhe, Germany) was dissolved in a small volume of ethanol and mixed with fish oil. The solutions containing different levels of lupinine were added to uncoated feed by vacuum-coating at concentrations of 0, 50, 75, 100, 250, 500, 1000, 5000 mg lupinine/kg of feed, respectively (Table 1). All feed were manufactured at The Centre for Feed Technology, The Norwegian University of Life Sciences, Ås, Norway.

### FISH, REARING CONDITIONS, AND SAMPLING

The feeding experiment was carried out in the fish research laboratory at the Norwegian University of Life Sciences (Ås, Norway). Rainbow trout with an average initial weight of 330 g, were randomly allocated (12 fish/tank) into sixteen circular fibreglass tanks (300 L) supplied with recirculated freshwater at a flow rate of 7.5 L/min and average temperature of 14°C. The feeding trial lasted sixty days and each diet was tested in duplicate tanks. Fish were fed twice daily by automatic band feeders set at 20% overfeeding based on expected feed intake. All individual fish were weighed at the start of the experiment, at day 20, at day 40 and at day 60. At the end of the trial, 9 fish from each tank was euthanised with a lethal concentration of tricaine methanesulfonate (MS 222). In addition to individual body weight, liver and spleen were also weighed. Three fish from each tank were randomly taken and stored at -22°C for whole body composition. Kidneys, spleens, brains, hearts, livers and middle and distal intestines were dissected from 3 fish per tank for histological examination. In addition, plasma and samples of intestinal content were collected (data not shown). Liver, muscle and brain tissue were also collected from 3 fish for alkaloid analyses and gene expression (data not shown).

### ANALYSES

Analysis of dry matter, crude protein (Kjeldahl N×6.25), and ash in diets and carcass were determined according to the methods of AOAC (2004). Crude fat was determined by weight difference after sample extraction with petroleum ether:acetone (80:20)

in an accelerated Solvent Extractor (ASE200; Dionex Corp., Sunnyvale, CA). Tissue samples for histology were fixed in 4% phosphate buffered formalin, dehydrated in ethanol, embedded in paraffin, pieced (4-6 µm) and stained with haematoxylin and eosin. Samples sections were evaluated for lesions under light microscope.

### STATISTICAL ANALYSIS

All data were analysed by linear regression with lupinine levels as the classification variable, using the Statistical Analysis System (SAS, 1990) with a significance level  $P \leq 0.05$ . A one-way ANOVA was also performed and significant difference between means was determined by Duncan's test.

### RESULTS AND DISCUSSION

Mortality was low throughout the entire experimental period, only disturbed by a biofilter fault at the middle of the experiment. Mortalities were not related to dietary treatments. Fish receiving the negative control (5000 mg lupinine/kg) was terminated for animal welfare reasons after 40 days, due to the poor feed intake and growth performance. Feed intake and growth rates of the rainbow trout declined in a linear manner in response to dietary inclusion of lupinine (Fig. 1). Additional analysis of variance confirmed a minimal effect on growth and feeding parameters by the inclusion of lupinine levels below 250 mg/kg.

The indole lupin alkaloid gramine showed to have a critical threshold, concerning growth and feed intake between 100 mg/kg and 500 mg/kg when fed rainbow trout (Glencross *et al.* 2006). The quinolizidine lupinine alkaloid, on the other hand, seems to affect these parameters in a somewhat lower concentration range (between 100 mg/kg and 250 mg/kg diet). Similar effect has been described in pigs and poultry, where the quinolizidine alkaloids showed greater influence on palatability than indole alkaloids (Pastuszewska *et al.* 2001).

Rainbow trout exhibited thus a similar range of sensitivity to the anti-palatability effects of alkaloids, compared to other vertebrate species. Tolerance concentration to dietary inclusion of quinolizidine alkaloids have been determined at up to 200 mg/kg diet for pigs (Godfrey *et al.* 1995), about 100 mg/kg diet for ducks (Olver and Jonker, 1998), 180 mg/kg diet for poultry (Olver and Jonker, 1997) and 330 mg/kg diets for rats (Robbins *et al.* 1996; Pothier *et al.* 1998). This is in agreement with our findings in the histology examination of liver tissue, which showed a slight reduction of fat droplets and glycogen stores in the hepatocytes by increasing levels of lupinine (Fig. 2). Depletion of glycogen and lipid stores in liver frequently occurs during fish starvation (Shearer, 1994). In this study however, there were no significant difference in the carcass composition that could be related to lupinine inclusion or starvation (Table 2).

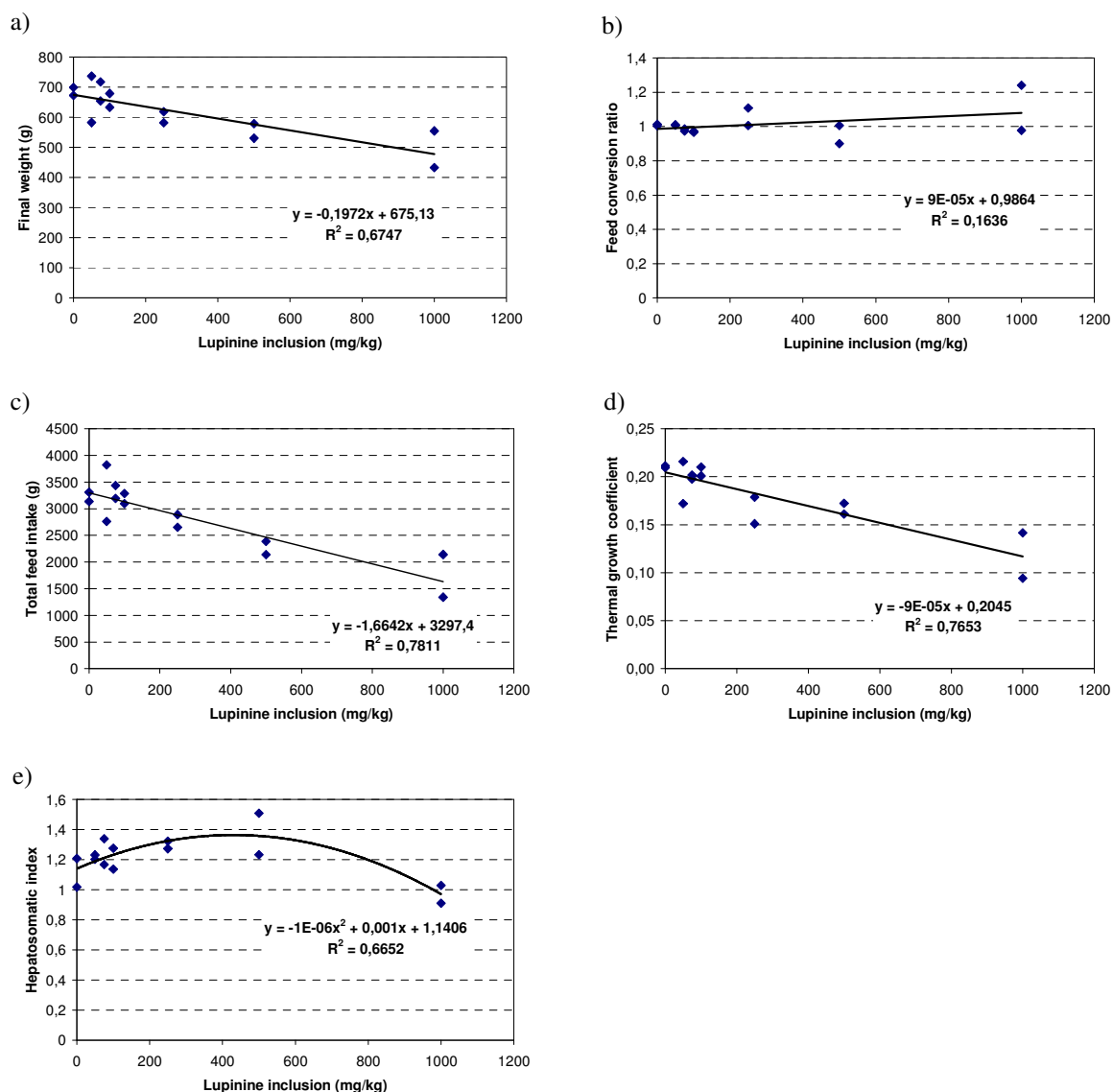
**Table 1.** Formulation and composition of experimental diets.

Diet Formulation g kg <sup>-1</sup>	Lupinine inclusion (mg kg <sup>-1</sup> )							
	0	50	75	100	250	500	1000	5000
Fish meal LT	724	724	724	724	724	724	724	724
Wheat starch	148	148	148	148	148	148	148	148
Lupinine (mg kg <sup>-1</sup> )	0	50	75	100	250	500	1000	5000
Vitamin, mineral and marker mixture	7	7	7	7	7	7	7	7
Fish oil	120	120	120	120	120	120	120	120
Proximate composition								
Dry matter (DM, g kg <sup>-1</sup> )	934	937	944	938	937	940	934	938
Crude protein (g kg <sup>-1</sup> DM)	510	502	500	503	503	510	501	498
Crude Fat (g kg <sup>-1</sup> DM)	173	170	175	176	184	173	177	182
Ash (g kg <sup>-1</sup> DM)	116	126	125	124	121	127	123	118

**Table 2.** Body composition and spleensomatic of rainbow trout fed with different experimental diets.

Diet	Lupinine inclusion (mg kg <sup>-1</sup> )							SEM	R <sup>2</sup>
	0	50	75	100	250	500	1000		
Dry matter (DM, g kg <sup>-1</sup> )	948.2 <sup>ab</sup>	954.7 <sup>a</sup>	945.5 <sup>b</sup>	949.6 <sup>ab</sup>	948.2 <sup>ab</sup>	947.6 <sup>b</sup>	945.9 <sup>b</sup>	1.95	-0.004
Crude protein (g kg <sup>-1</sup> DM)	551.4 <sup>a</sup>	490.6 <sup>c</sup>	495.6 <sup>bc</sup>	553.4 <sup>a</sup>	541.0 <sup>ab</sup>	536.1 <sup>abc</sup>	541.9 <sup>ab</sup>	13.56	0.013
Crude Fat (g kg <sup>-1</sup> DM)	370.4 <sup>cb</sup>	435.4 <sup>a</sup>	399.4 <sup>ab</sup>	349.4 <sup>c</sup>	383.8 <sup>abc</sup>	350.5 <sup>bc</sup>	374.8 <sup>abc</sup>	17.99	-0.018
Ash (g kg <sup>-1</sup> DM)	58.1	65.8	41.9	53.0	73.7	46.8	58.4	14.37	-0.002
SSI	0.09	0.10	0.10	0.08	0.08	0.09	0.09	0.01	0.000

Means scored with different letters are significantly different (n = 2, P < 0.05).



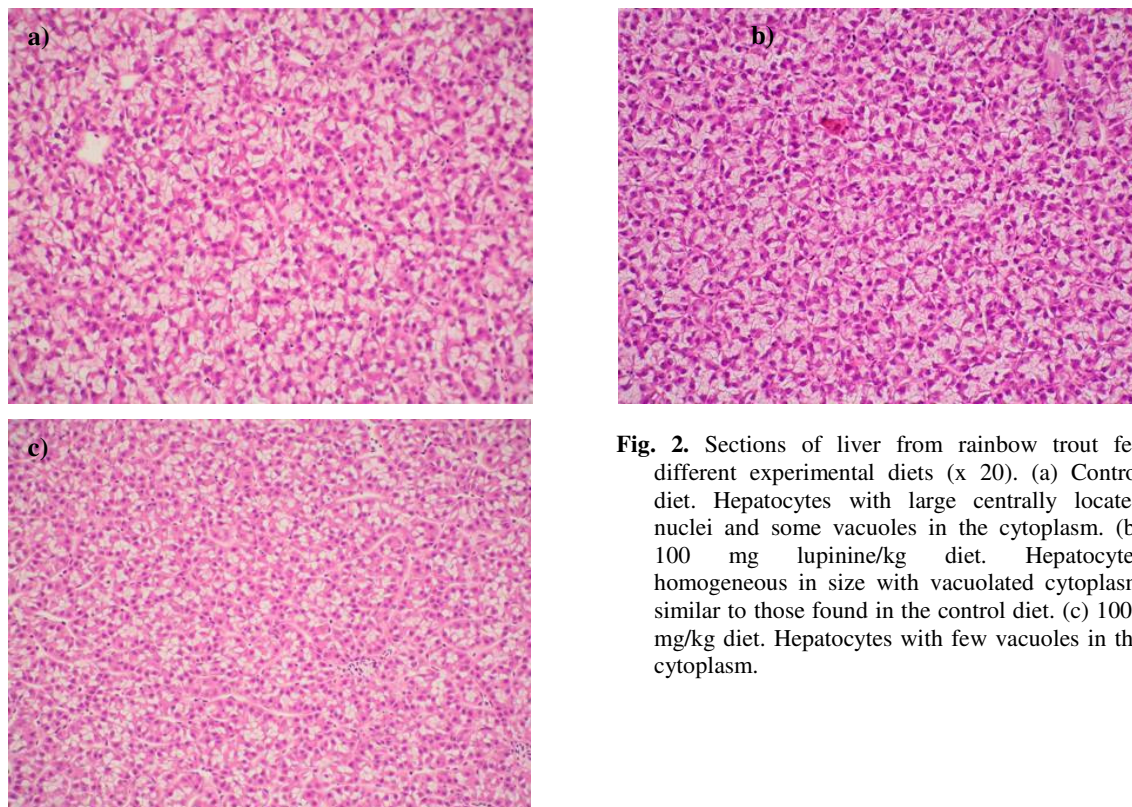
**Fig. 1.** Relationship between lupinine inclusion levels and growth (a), feed conversion ratio (b), feed intake (c), TGC (d), Hepatosomatic index (e).

Despite the toxic effects of lupin alkaloids that have been described at organ and cellular level (Wink, 1998; Wink *et al.* 1998), the trout displayed no evident lesion in the middle and distal intestine, kidney, heart or liver when fed lupinine alkaloid.

Absence of any histological evidence for toxic effect of gramine in trout has also been observed previously by Glencross *et al.* (2006). These results suggest that rainbow trout may be able to tolerate lupin alkaloid to a higher extent than other vertebrates. Experiments with pigs, rats and poultry have shown that high amount of gramine can produce parenchymatous and vacuolar degeneration of hepatocytes, focal necrosis of epithelium of renal tubules and focal hyaline degeneration of heart muscle fibres (Pastuszewska *et al.* 2001). Inflammatory lesions were also seen in

hepatocytes of rats fed diets containing 250-5000 mg quinolidizine alkaloid/kg diets (Butler *et al.* 1996). However, none of these histopathological conditions were observed at any lupinine inclusion level with rainbow trout.

In conclusion, the lupinine alkaloid had a clear palatability reducing effect on rainbow trout at inclusion levels above 100 mg/kg diet. This level is higher than the alkaloid content found in sweet lupin varieties commonly used in fish feed formulations. Correspondingly, it is plausible to also include some bitter yellow lupin varieties in fish feed providing some regard is made to the critical threshold limit of 100 mg/kg. Further research to evaluate the effect of other lupin quinolidizine alkaloids in fish would be of value to examine the effect of different alkaloid varieties.



**Fig. 2.** Sections of liver from rainbow trout fed different experimental diets (x 20). (a) Control diet. Hepatocytes with large centrally located nuclei and some vacuoles in the cytoplasm. (b) 100 mg lupinine/kg diet. Hepatocytes homogeneous in size with vacuolated cytoplasm similar to those found in the control diet. (c) 1000 mg/kg diet. Hepatocytes with few vacuoles in the cytoplasm.

### LITERATURE CITED

- AOAC (Association of Official and Analytical Chemists), 1998. Official methods of analysis, 16th edn. Assoc. Anal. Chem, Washington, DC, USA.
- Butler, W.H., G.P. Ford and D.M. Creasy. 1996. A 90-day feeding study of lupin (*Lupinus angustifolius*) flour spiked with lupin alkaloids in the rat. *Food and Chemical Toxicology* 34: 531-536.
- delaVega, R., M.P. Gutierrez, C. Sanz, R. Calvo, L.M. Robredo, C. delaCuadra and M. Muzquiz. 1996. Bactericide-like effect of Lupinus alkaloids. *Industrial Crops and Products* 5: 141-148.
- Francis, G., H.P.S. Makkar and K. Becker. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199: 197-227.
- Glencross B., J. Curnow, W. Hawkins and M. Felsing. 2002. Evaluation of yellow lupin *Lupinus luteus* meal as an alternative protein resource in diets for sea-cage reared rainbow trout *Oncorhynchus mykiss*. *Journal of World Aquaculture Society* 33: 287-296.
- Glencross, B., D. Evans, W. Hawkins and B. Jones. 2004. Evaluation of dietary inclusion of yellow lupin (*Lupinus luteus*) kernel meal on the growth, feed utilisation and tissue histology of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 235: 411-422.
- Glencross, B., D. Evans, N. Rutherford, W. Hawkins, P. McCafferty, K. Dods, B. Jones, D. Harris, L. Morton, M. Sweetingham and S. Sipsas. 2006. The influence of the dietary inclusion of the alkaloid gramine, on rainbow trout (*Oncorhynchus mykiss*) growth, feed utilisation and gastrointestinal histology. *Aquaculture* 253: 512-522.
- Glencross, B.D., M. Booth and G.L. Allan. 2007. A feed is only as good as its ingredients – a review of ingredient evaluation strategies for aquaculture feeds. *Aquaculture nutrition* 13: 17-34.
- Godfrey N.W., A.R. Mercy, Y. Emms and H.G. Payne. 1985. Tolerance of growing-pigs to lupin alkaloids. *Australian Journal of Experimental Agriculture* 25: 791-795.
- Korper, S., M. Wink and R.H.A. Fink. 1998. Differential effects of alkaloids on sodium currents of isolated single skeletal muscle fibres. *Febs Letters* 436: 251-255.
- Olver, M.D. and A. Jonker. 1997. Effect of sweet, bitter and soaked micronised bitter lupins on broiler performance. *British Poultry Science* 38: 203-208.
- Olver, M.D. and A. Jonker. 1998. Effects of sweet, bitter and soaked micronised bitter lupins on duckling performance. *British Poultry Science* 39: 622-626.
- Pastuszewska, B., S. Smulikowska, J. Wasilewko, L. Buraczewska, A. Ochtabinska, A. Mieczkowska, R. Lechowski and W. Bielecki. 2001. Response of animals to dietary gramine. I. Performance and selected haematological, biochemical and histological parameters in growing chicken, rats and pigs. *Archives of Animal Nutrition-Archiv Fur Tierernahrung* 55: 1-16.
- Petterson, D.S., S. Sipsas and J.B. Mackintosh. 1997. The chemical composition and nutritive value of Australian pulses. Grains Research and Development Corporation, Canberra, Australia. 65 pp.
- Pothier J., S.L. Cheav, N. Galand, C. Dormeau and C. Viel. 1998. A comparative study of the effects of sparteine, lupanine and lupin extract on the central nervous system of the mouse. *Journal of Pharmacy and Pharmacology* 50: 949-954.

- Robbins, M.C., D.S. Petterson and P.G. Brantom. 1996. A 90-day feeding study of the alkaloids of *Lupinus angustifolius* in the rat. *Food and Chemical Toxicology* 34: 679-686.
- SAS. 1990. Statistical Analysis System, User's guide. Version 6, 4th edition. SAS institute, Cary, NC, USA. 956 pp.
- Shearer, K.D. 1994. Factors affecting the proximate composition of cultured fishes with emphasis on salmonoids. *Aquaculture* 119: 63-88.
- Sujak, A., A. Kotlarz and W. Strobel. 2006. Compositional and nutritional evaluation of several lupin seeds. *Food Chemistry* 98: 711-719.
- Tacon, A. 1997. Fishmeal Replacers: Review of antinutrients within oilseeds and pulses, a limiting factor for aquafeed green revolution? pp. 153-182. *IN* A. Tacon and B. Basurco (eds.). *Feeding tomorrow's fish*, vol. 22. Editions Cahiers options Méditerranéennes, CIHEAM. Spain.
- Wink, M. 1998. Modes of action of alkaloids. pp. 301-326. *IN* M.F. Roberts and M. Wink (eds.). *Alkaloids: Biochemistry, ecology and medicinal applications*. Plenum, New York.
- Wink, M., C. Meißner and L. Witte. 1995. Patterns of quinolizidine alkaloids in 56 species of the genus *Lupinus*. *Phytochemistry* 38: 139-153.
- Wink, M., T. Schmeller and B. Latz-Bruning. 1998. Modes of action of allelochemical alkaloids: Interaction with neuroreceptors, DNA, and other molecular targets. *Journal of Chemical Ecology* 24: 1881-1937.