



Studi sull'effetto ansiolitico e miglioramento dei processi di memorizzazione dello Stabilium 200



Questo è un documento di sintesi tradotto in italiano dello studio effettuato sulla efficacia dello Stabilium 200.

Di seguito il documento integrale in lingua inglese

### **PREMESSA**

Lo Stabilium 200\* contiene Garum Americum \* (GA), che è un autolisato proteico del pesce Blue Ling (*Molva dyptergia*). Tale autolisato si caratterizza per la presenza di elevati livelli di peptidi piccoli e di circa il 30,7% di amminoacidi liberi come l'acido glutammico, la glicina, l'acido aspartico, la leucina, la tirosina, la valina, la serina ed il triptofano. Per quanto riguarda i grassi, il 30% di questi è costituito da acidi grassi polinsaturi, tra cui gli omega 3 EPA e DHA. Inoltre, il GA contiene le vitamine A, D, E, B1, B2, B3, B6 e B12, e i minerali magnesio, fosforo, selenio e iodio.

### **SCOPO DELLO STUDIO**

Lo studio si proponeva di valutare (a) l'effetto ansiolitico e (b) nootropico (miglioramento dei processi di memorizzazione) dello Stabilium 200\* .

### **PROCEDURA**

Sono stati applicati protocolli sperimentali validati in modelli animali, quali l'esplorazione di aree potenzialmente pericolose ( a ) e lo spegnimento di una luce avversa (b).

Per valutare l'effetto ansiolitico, 32 ratti sono stati divisi in 4 gruppi: controllo, diazepam (DZP, 3mg/kg/giorno), GA (25 e 50 mg/kg/giorno). Tali prodotti sono stati somministrati in due dosi distinte (alle ore 9 e 15) nei tre giorni precedenti il test; al quarto giorno, i ratti hanno assunto l'intera quantità in una unica dose.

Nel test di estinzione della luce avversa, 60 ratti sono stati divisi in 5 gruppi: controllo, piracetam (nootropico, 300 mg/kg/giorno) e GA (25,50, 100 mg/kg/giorno). Il trattamento è durato 8 giorni. Nei giorni 9 e 10 sono stati effettuati i tests.

### **RISULTATI**

Il trattamento con entrambe le dosi di GA ha determinato un aumento significativo, rispetto al controllo, dell'ingresso e permanenza nell'area "avversa". Tale effetto è risultato confrontabile con quello della diazepam (noto ansiolitico).

Per quanto riguarda il test (b), il trattamento con GA ha determinato , rispetto al controllo, un maggior numero di pressioni sulla leva di spegnimento della luce avversa. Tali effetti sono paragonabili a quelli prodotti dal piracetam solo nel caso della dose di GA più alta (100 mg/kg/giorno).

### **CONCLUSIONI**

Lo studio evidenzia che il GA, cioè lo Stabilium 200\*, ha proprietà ansiolitiche e può accelerare i processi di memorizzazione.



## THE EFFECTS OF GARUM ARMORICUM® (GA) ON ELEVATED-PLUS MAZE AND CONDITIONED LIGHT EXTINCTION TESTS IN RATS

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**ABSTRACT:** *Garum Armoricum® (GA), a compound rich in polyunsaturated fatty acids, free amino acids, small peptides, vitamins and minerals, was evaluated on two fear-related assays in rats. GA and diazepam (DZP) increased entries into open arms relative to placebo, as well as percentage of open arm entries in the elevated plus-maze test. In a similar fashion, all drugged groups spent more time inside the open arms and less time inside the enclosed arms. After a two-day period of conditioned avoidance learning of an aversive bright light, GA and vehicle groups successfully discriminated the active from the inactive lever. On the initial day of acquisition, GA and piracetam (PIR) groups achieved successful discrimination though the control group did not. These results indicate that GA may have anxiolytic-like effects without causing learning deficiencies. These psychotropic properties of GA may be due to the synergistic action of its active constituents.*

**KEYWORDS:** Anxiety, Cognition, Elevated plus-maze, Garum Armoricum®, Light extinction test, Rat

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### INTRODUCTION

In the last few years, several natural substances have been introduced with potential to combat age-related oxidative stress and neural dysfunction, including casein hydrolysates extracted from milk (Messaoudi et al. 2005; Violle et al. 2006) and fish

protein hydrolysates (Bernet et al. 2000). The list may be extended when considering vitamins A, C, and E with selenium or zinc (Gray et al. 2003; Morris et al. 2002; Sies and Stahl, 1995) beta-carotene (Hu et al. 2006), polyphenols (Bisson et al. 2008; Rozan et al. 2007; Schroeter et al. 2000), and essential omega-3 fatty acids (Morris et al. 2005; Saugstad, 2006). Supplementation with n-3 long-chain polyunsaturated fats, docosahexaenoic acid (DHA) and ethyl-eicosapentaenoic acid (EPA), has been proposed to reduce the risk of a variety of diseases (Assisi et al. 2006). But the extent to which DHA and EPA supplementation is beneficial in counteracting age-related cognitive decline and anxiety disorders is largely an open question. An EPA supplemented diet attenuated stress/anxiety and inflammatory responses caused by subchronic intracerebroventricular injections of interleukin-1 $\beta$  in rats (Song et al. 2003). A fish-oil diet increased exploratory activity in young mice, but not in mature and old mice (Carrié et al. 2000a). More thorough experimentation has been accomplished with n-3 fatty acid deficient diets, sometimes causing effects on cerebral activation and anxiety as measured in open-field and elevated plus-maze tests, as well as learning disabilities (Fedorova and Salem 2006). For example, rats weaned by n-3 fatty acid deficient dams had poorer olfactory and spatial learning (Greiner et al. 1999).

Because of the limited benefits often observed after administration of single substances (Grundman and Delaney 2002), it is of interest to explore compounds with synergistic potential. In this perspective, we examined the effects of Garum Armoricum®, an enzymatic autolysate from the Blue Ling fish (*Molva dypterygia*), rich in polyunsaturated fatty acids and vitamins A, B and E as well as magnesium and selenium, on two fear-related assays in rats. In the elevated plus-maze test (Cole and Rodgers 1993; Pellow et al. 1985), two wall-enclosed arms

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are opposite each other beside two arms without walls. Open arm entries and duration reflect exploration of a potentially dangerous area relative to safer enclosed arms. The well-characterized benzodiazepine anxiolytics are known to increase open arm exploration in mice (Cole and Rodgers 1993) and rats (Pellow et al. 1985). In the light extinction test (Benton et al. 2003; Messaoudi et al. 1996), rats are briefly trained to press a lever causing extinction of an aversive bright light. This test was sensitive to heat stress, an effect reversed by a cocoa polyphenolic extract (Rozan et al. 2007). *Garum Armoricum* was compared with piracetam, a nootropic drug facilitating memory processes in many types of tasks (Winblad 2005).

## MATERIALS AND METHODS

### *Animals*

Male Wistar/Han rats (HsdBrHn, Harlan, The Netherlands) weighing 250-275 g were housed in groups of four inside 48x27x20 cm polycarbonate cages (UAR, Epinay-sur-Orge, France) under stable conditions with respect to humidity (50±5%) and temperature (22±1°C). The rats were maintained on a 12 h light/12 h dark cycle (on at 20.00 pm-08.00 am). Food pellets (Harlan, Gannat, France) and tap water were provided at all times. After a 7-day adaptation period, the rats were weighed and randomly assigned to four treatment groups (n=8) for the elevated plus-maze test and five other groups (n=12) for the light extinction test.

The experiments adhered to guidelines provided by the ASAB Ethical Committee for the use of animals in research (Anim. Behav. 2006, 71, 245-254) and the Canadian Council on Animal Care (Guide to the Care and Use of Experimental Animals), 2<sup>nd</sup> ed., vol.2 (1993); 1<sup>st</sup> ed., vol. 1 (1984). All procedures were also in compliance with the rules provided by the European Community Council Directive of 24 November 1986 (86/609/EEC).

### *Elevated plus-maze*

The elevated plus-maze was constructed from plasticized wood with two open (50x12 cm) and two enclosed arms (wall height: 40 cm) placed on opposite sides at a height of 80 cm. The four arms extended from a common central platform (12x12 cm). The rat was placed in the centre with its head turned towards an open arm and was left to explore the apparatus in a single 5-min session. The test was conducted under dim white light and behaviours recorded with a video camera. Videotapes were subsequently analyzed by a trained observer unaware of treatment variables. The number of entries (4-paw criterion) and the time spent inside each arm were measured. The percentage of entries and the percentage of duration were then calculated, as defined by open arm entries divided by open + enclosed entries and open arm duration divided by open + enclosed arm durations.

### *Conditioned light extinction*

The light extinction apparatus (TESLA®, ETAP/Intellibio, France) consisted of a chamber (50x40x37 cm) with a light level maintained at 1200 lux. Two levers were included: an active one causing a 30-s period of darkness whenever pressed down and an

inactive one. During the 20-min acquisition phase and the retest session conducted on the following day, the rats were continuously exposed to the brightly lit chamber unless an active barpress occurred. The number of active and inactive lever presses in the light period was recorded, as well as total lever presses in both light and dark periods.

### *Products and preparation of the enzymatic fish protein autolysate*

GA (Stabilium®200 containing *Garum Armoricum*®) was provided by CGD-Yalacta Laboratories, France. Diazepam (DZP) was purchased from Sigma-Aldrich, France and Piracetam (PIR) from UCB Pharma S.A., France. *Garum Armoricum*® is produced by controlled enzymatic autolysis of the Blue Ling fish proteins (*Molva dypterygia*). The fish protein autolysate contains a high concentration of small peptides and 30.7% of free amino acids, including glutamic acid, glycine, aspartic acid, leucine, tyrosine, valine, serine, and tryptophan. Thirty percent of fats are essential polyunsaturated fatty acids (PUFA), including omega-3 PUFA, DHA and EPA and omega-6 PUFA. In addition GA is rich in vitamins A, D, E, B1, B2, B3, B6 and B12, and in minerals (i.e. magnesium, selenium, phosphorus and iodine). All substances were suspended in a 0.5% methylcellulose aqueous solution and orally administered in a volume of 5 ml/kg BW. Control rats received a 5 ml/kg BW methylcellulose solution as the vehicle under identical conditions.

In the elevated plus-maze test, 32 rats were divided into four groups: control, DZP (3 mg/kg/day), and GA (25 and 50 mg/kg/day). The products were administered twice per day (9 am and 3 pm) during 3 days preceding the test session. On day 4, the rats were given the entire dose and evaluated 1 h later during the first 3 h of the dark period.

In the conditioned light extinction test, 60 rats were divided into five groups: control, piracetam (300 mg/kg/day), and GA (25, 50 and 100 mg/kg/day). The products were administered twice per day for 8 days preceding the test session. On days 9 and 10, the rats were given the entire dose and evaluated 1 h later during the first 3 h of the dark period.

### *Statistical analyses*

The effects of treatments were analyzed with one-way analysis of variance (ANOVA), followed by Dunnett's t-test, for comparisons of each experimental group to control. The paired t-test (2-tail.) was used to compare active with inactive lever presses in each group. Results are expressed as mean ± SEM. Differences are considered to be significant at  $p < 0.05$ . All statistical analyses were carried out with Statview® 5 software (SAS, Institute Inc., Carey, USA).

## RESULTS

### *Elevated plus-maze*

There were significant group differences for entries into open arms ( $F(3,28) = 6.7, p = 0.002$ ), enclosed arms ( $F(3,28) = 3.44, p = 0.03$ ), and percentage of entries into open arms ( $F(3,28) = 23.93, p < 0.0001$ ). As shown in Table I, both doses of GA and the single dose of DZP increased entries into open arms relative to placebo, as well as percentage of open arm entries. In

contrast, enclosed arm entries were reduced by the lower dose of GA and by DZP.

The pattern was similar in regard to arm duration. Indeed, group differences were found for open arm duration ( $F(3,28) = 7.9, p < 0.001$ ), enclosed arm duration ( $F(3,28) = 6.67, p = 0.002$ ), and open/total arm duration ( $F(3,28) = 6.8, p = 0.001$ ). Relative to placebo, all three drugged groups spent more time inside the open arms and less time inside the enclosed arms (see Table I).

#### Conditioned light extinction test

After two-day training in light extinction test, the vehicle group successfully discriminated the active from the inactive lever on the basis of higher lever presses on the former during the light period (paired t-test,  $p < 0.01$ ). Likewise, there were more active lever presses in rats receiving GA at the doses of 25, 50 and 100 mg/kg ( $p < 0.05, p < 0.01$  and  $p < 0.01$ , respectively) (see Figure 1).

FIGURE 1. The number of presses on active (hatched columns) and inactive (white columns) levers during the light period causing light extinction or not in rats given Garum Armoricum (GA) and piracetam (PIR) or vehicle (VEH) during day 2 of training in the conditioned light extinction test. \* $p < 0.05$ ; \*\* $p < 0.01$  (Paired t-test: AL vs. IL). Values represent mean  $\pm$  SEM,  $n = 12$ .

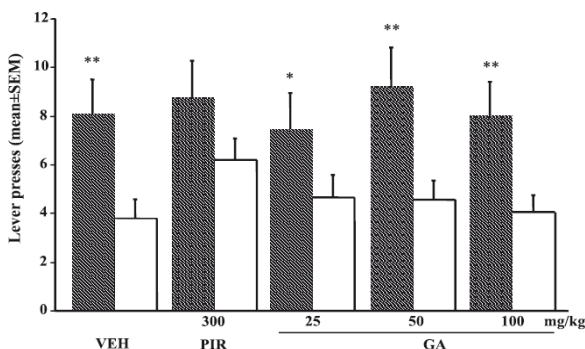


FIGURE 2. The number of presses on active (AL) (hatched columns) and inactive levers (IL) (white columns) during the light period causing light extinction or not in rats given Garum Armoricum (GA), piracetam (PIR) or vehicle (VEH) during day 1 of training in the conditioned light extinction test \* $p < 0.05$ ; \*\* $p < 0.01$  (Paired t-test: AL vs. IL). Values represent mean  $\pm$  SEM,  $n = 12$ .

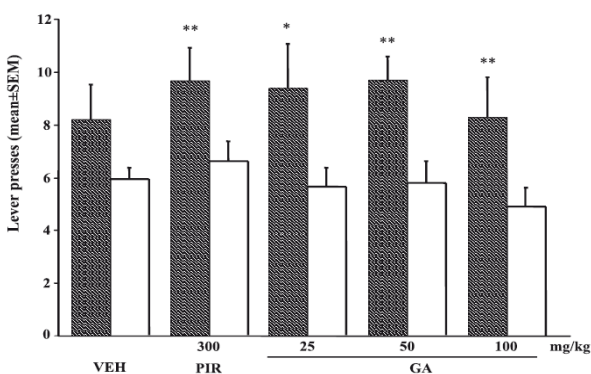


TABLE 1. Exploratory activity of rats orally treated with Garum Armoricum (GA), diazepam (DZP) and vehicle (VEH) in a single 5-min session of the elevated plus-maze test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. Vehicle (Dunnett's t-test). Values represent mean  $\pm$  SEM,  $n = 8$ .

MEASURES	VEH	DZP 3 mg/Kg	GA 25 mg/Kg	GA 50 mg/Kg
Arm entries				
Open	1.6 $\pm$ 0.5	5.4 $\pm$ 0.6***	4.5 $\pm$ 0.6**	4.3 $\pm$ 0.8**
Enclosed	7.5 $\pm$ 0.8	5.1 $\pm$ 0.6*	4.9 $\pm$ 0.4**	6.1 $\pm$ 0.7
Open/total (%)	14.9 $\pm$ 4.2	51.2 $\pm$ 2.0***	47.4 $\pm$ 1.8***	39.6 $\pm$ 4.5***
Arm duration (s)				
Open	23.8 $\pm$ 8.6	72.6 $\pm$ 9.1***	74.9 $\pm$ 5.8***	65.8 $\pm$ 10.0**
Enclosed	217.9 $\pm$ 11.1	140.5 $\pm$ 14.1***	146.8 $\pm$ 10.6***	154.8 $\pm$ 18.2**
Open/total (%)	9.9 $\pm$ 3.6	34.8 $\pm$ 4.9***	34.2 $\pm$ 3.2***	31.2 $\pm$ 5.8**

Moreover, GA-treated groups, at the three doses, had more active lever presses on day 1 of acquisition ( $p < 0.05, p < 0.01, p < 0.01$ , respectively), at a time when the results with the control group approached, but did not reach significance ( $p = 0.07$ ) (see Figure 2).

The results were mixed with respect to PIR, lever discrimination being successful on day 1 ( $p < 0.01$ ), but not on day 2 ( $p = 0.09$ ). As seen in Table II, there were no group differences in total lever presses during light and dark periods on day 1 ( $F(4,55) = 0.69, p > 0.05$ ) or day 2 ( $F(4,55) = 0.63, p > 0.05$ ).

TABLE 2. Total lever presses in light and dark periods of rats orally treated with Garum Armoricum (GA), piracetam (PIR), and vehicle (VEH) on days 1 and 2 of testing. Values represent mean  $\pm$  SEM,  $n = 12$ .

VEH	PIR 300 mg/Kg	GA 25 mg/Kg	GA 50 mg/Kg	GA 100 mg/Kg
Day 1				
26.8 $\pm$ 4.1	40.2 $\pm$ 5.8	35.3 $\pm$ 6.9	38.2 $\pm$ 4.8	35.4 $\pm$ 8.3
Day 2				
21.7 $\pm$ 4.1	30.9 $\pm$ 5.2	23.9 $\pm$ 5.7	31.2 $\pm$ 6.3	29.6 $\pm$ 6.0

## DISCUSSION

Relative to placebo, rats receiving GA by the oral route had higher entries into open arms and open arm duration in the elevated plus-maze. These effects were also observed with DZP, a benzodiazepine with well-known anxiolytic properties in several tests including the elevated plus-maze (Cole and Rodgers 1993; Pellow et al. 1985).

To our knowledge, this is the first study on the effects of PUFA supplementation on the elevated plus-maze. Variable results in this test have been obtained with diets deficient in n-3 fatty acids (Fedorova and Salem 2006). Relative to normal diets, n-3 fatty acid deficiencies in mice decreased open arm duration in one study (Carrié et al. 2000a) but had no effect in two others (Frances et al. 1995; Nakashima et al. 1993). In rats, open arm duration decreased in one study (Takeuchi et al. 2003) and was unchanged in another (Belzung et al. 1998). The anxiety level may be an important factor in mediating variable outcomes, as different results were obtained under different lighting conditions, with diet-induced increases of anxiety found only under bright illumination (Fedorova and Salem 2006). The age factor may also

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be important on the basis of age-dependent effects in the open-field induced by n-3 fatty acid supplementation (Carrié et al. 2000b). It remains to be determined whether age-dependent effects are found with our substance in this and other tests of exploratory activity.

After two-day training in light extinction test, the vehicle group had a higher number of active relative to inactive lever presses. Likewise, at all doses, there were more active lever presses in rats receiving GA. The GA groups had more active lever presses as early as day 1, at a time when the results with the control group only approached significance. Although based on a single data point, these results indicate that GA may accelerate learning processes.

The results were different with respect to PIR, a modulator of cholinergic and glutamatergic synapses, known to facilitate acquisition in many tasks (Winblad 2005). Indeed, rats given PIR successfully discriminated levers on day 1 but not on the following day, due to a higher number of inactive lever presses. The facilitation on the initial day confirms a previous finding with PIR on acquisition of a bar-pressing response under a continuous reinforcement schedule (Krejci and Dlabac 1984). Although once again based on a single data point, these results indicate that in the present task, PIR may not facilitate long-term memory processes.

In conclusion, we found that *Garum Armoricum*<sup>®</sup> has anxiolytic-like properties. A more widespread battery of anxiety tests is necessary to determine whether this supplement rich in various active constituents may ease fear not only in the absence of amnesia, but with potential for facilitating learning and memorization.

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