Effect of larval diapause on sex pheromone secretion by female *T. granarium*

| Female age (days) | No. of males responding[a] to pheromone extract from post-diapause females I | II | III | normal females I | II | III |
|---|---|---|---|---|---|---|
| 1–2 | 8.7 | 6.0 | 8.3 | 9.0 | 8.7 | 8.8 |
| 3–4 | 8.2 | 7.2 | 7.0 | 8.7 | 7.6 | 8.5 |
| 5–7 | 8.2 | 8.4 | 8.5 | 7.6 | 8.0 | 8.4 |
| 8– 9 | 8.8 | 8.2 | 9.4 | 6.7 | 6.0 | 5.7 |
| 10–11 | 8.1 | 7.9 | 7.8 | 4.8 | 6.6 | 6.2 |
| 12–14 | 8.0 | 8.4 | 8.7 | – | – | – |
| 15–16 | 6.6 | 5.8 | 5.9 | – | – | – |
| 17–18 | 5.2 | 5.1 | 5.9 | – | – | – |

[a] Each figure represents mean of 10 assays. I, II, III: Pheromone extracted from 3 batches of 50 females each.

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The degree of response of post-diapause males was also significantly higher than that of normal males (figure). The enhanced responsiveness of diapause males was evident even within the first minute of observation; and this difference was discernible throughout the period of assay. It is therefore apparent that the diapause males are able to detect the presence of pheromone more quickly. It may be due to the enhanced agility of the post-diapause males as a result of increased energy reserves. Also the possibility of lowered threshold for pheromone perception cannot be ruled out. This, and the fact that such diapause permits enhanced nutrient reserves, tends to suggest that these insects may have greater mating vigour than normal ones. Enhanced mating vigour as a result of diapause has been observed in alfalfa weevil[9].

The results presented here clearly indicate that induction of larval diapause by sub-optimal temperature, enhances the efficiency of pheromone communication between the sexes. In *T. granarium* diapause is also induced by factors like crowding, presence of faecal matter in the medium, and sub-optimal nutrition[10,11]. However, it is not known whether larval diapause induced by these factors will lead to enhanced pheromone secretion and male response.

## Effects of high hydrostatic pressures on the movements of $Na^+$, $K^+$ and $Cl^-$ in isolated eel gills

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*Summary.* Hydrostatic pressure applied to isolated eel gills induces changes in the tissue $Na^+$, $K^+$ and $Cl^-$ contents. It also inhibits the activity of the ($Na^+ + K^+$) ATPase. Results are discussed in terms of an effect of pressure on the $Na^+$ and $Cl^-$ pumps and on the passive permeability processes.

When isolated gills from sea water eels are incubated in oxygenated sea water, a constant level of NaCl is maintained against the diffusion gradient[2–5]. This seems to result from an active NaCl extrusion coupled with $K^+$ entry. The maintenance of this constant ionic level indeed is very sensitive to the presence of oxygen in the medium and to the action of specific inhibitors like ouabain and 2–4(α)-dinitrophenol[4–6]. Nevertheless, the connections between different transport processes, as well as the real nature of the active mechanisms, are still unknown.

Most of the theories on ionic passive and active transfers involve the existence of chemical reactions, ionization processes, binding to carriers or enzymes. These chemical processes may be associated with volume changes and may therefore be sensitive to hydrostatic pressure[7]. Hence high pressure could be utilized as an analytical tool to approach the study of the structure of the cell membrane in relation to function. From this point of view, pressure has already been shown to act selectively on passive ionic permeability and to inhibit the $Na^+$ active transport of the isolated frog skin[8–10]. Such results led us to study the effects of high hydrostatic pressure on the movements of $Na^+$, $K^+$ and $Cl^-$ in isolated gills of the eel.

*Material and methods.* Experiments have been performed on isolated gills from silver eels Anguilla anguilla L. adapted to sea water (521 mEq/l $Na^+$, 19 mEq/l $K^+$, 610 mEq/l $Cl^-$, pH 7.8, t° 15 °C). The isolated tissues have been submitted for 1 h to hydrostatic pressures in an apparatus designed to avoid the presence of any gas phase which has been described elsewhere[8]. In the present study, the volume of preoxygenated saline used in the experimental set-up was 50 ml. Dissection of the gills as well as incubation conditions have been described previously[5].

At the end of the experiments, gill filaments are cut off the incubated gills; they are blotted on filter paper, weighed and dried at constant weight in an oven at 110 °C for dry weight measurements. The gill filaments are then digested for 48 h in 4 ml $HNO_3$ 0.1 N. Tissue ionic concentrations have been calculated from the measurements of the ions content in the digestion medium. $Na^+$ and $K^+$ determinations were done by flame photometry. $Cl^-$ content was estimated with a Buchler-Cotlove chloridometer. The results, calculated in μEq of ion per g tissue dry weight, have been expressed in this report as ratio of the

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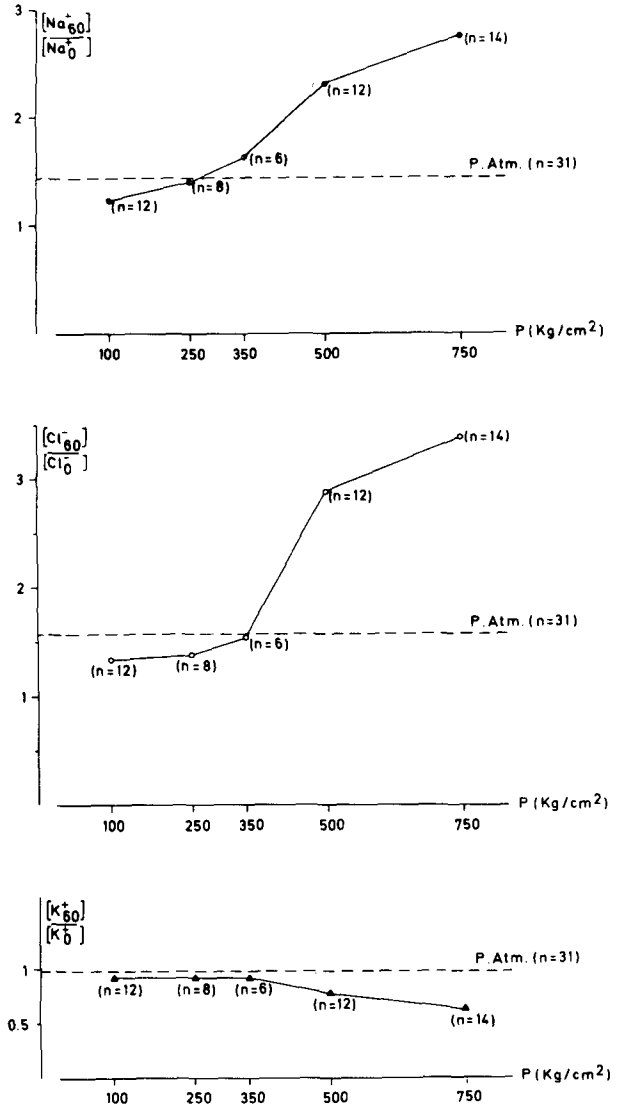
| ATPase activity of gill extract from sea water adapted eels* at 1 Atm (≈ 1.03 kg/cm²) | | Effects of high hydrostatic pressures on the activity of the ($Na^+$ + $K^+$) ATPase | |
|---|---|---|---|
| | | Pressure in kg/cm² | % variation due to pressure |
| ($Mg^{++}$, $Na^+$ + $K^+$) ATPase | 0.65 ± 0.02 (n = 6) | 100 | *no significant effect* |
| $Mg^{++}$ ATPase (ouabaine non sensitive) | 0.34 ± 0.01 (n = 6) | 250 | − 25.5 |
| ($Na^+$ + $K^+$) ATPase (ouabain sensitive) | 0.31 ± 0.01 (n = 6) | 500 | − 52.8 |
| % ($Na^+$ + $K^+$) ATPase | 47.4 ± 0.5 (n = 6) | 750 | − 71.9 |
| ($Mg^{++}$, $Na^+$ + $K^+$) ATPase / $Mg^{++}$ ATPase | 1.90 ± 0.02 (n = 6) | 1000 | − 83.7 |

*Incubation medium: ATP 4 mM, NaCl 100 mM, EGTA 0.25 mM
$MgCl_2$ 5 mM, KCl 25 mM, Tris buffer 25 mM
(+ ouabain 0.2 mM when necessary)

Results are expressed as mean ± S. E. M., *in μMPi/mg membrane protein/h.*




Effects of hydrostatic pressure on the $Na^+$, $K^+$ and $Cl^-$ content of the eel isolated gills. Ionic concentrations are expressed as the ratio between the ion content of the tissue after ($Na^+_{60}$, $K^+_{60}$ or $Cl^-_{60}$) and before ($Na^+_0$, $K^+_0$ or $Cl^-_0$) 60 min at elevated pressure. The doted line gives the ionic level reached when gills are incubated at atmospheric pressure without oxygenation in the experimental set-up. (n) represents the number of experiments. S.E.M. are always lower than ± 0.23.

ionic content measured before and after incubation under hydrostatic pressure.

The membrane ATPase extracts have been prepared from isolated gill filaments according to the method previously described[8]. The total ATPase activity was determined in presence of 4 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 25 mM KCl, 0.25 mM EGTA, Tris buffer 25 mM pH 7.4. After incubation at 22 °C for 30 min at the atmospheric pressure or under high hydrostatic pressures, the reaction was stopped by adding 0.2 ml of 50% trichloroacetic acid. Inorganic phosphate was determined by the method of Fiske and Subbarow[11]. Protein was determined according to the method of Lowry et al.[12]. ($Na^+$ + $K^+$)ATPase (ouabaine sensitive) was calculated as the difference between the total activity and the part remaining when 0.2 mM ouabain was added to the medium. Activity was expressed in μM Pi/(mg membrane protein) · h.

*Results and discussion.* The effects of pressure steps up to 750 kg/cm² on the $Na^+$, $K^+$ and $Cl^-$ contents of isolated gills are summarized in the figure. First of all, it can be seen that incubation at atmospheric pressure in our experimental set-up, where no oxygenation can be provided, leads to an increase in the $Na^+$ and $Cl^-$ tissue content. We have previously shown[5] that the method of supplying oxygen to the tissue in vitro determines its ionic steady state levels. The level reached with oxygen was, for example, lower than with air. In the present experimental conditions, the increase therefore only reflects the transient variations in ionic content leading to another specific steady-state level.

Application of pressure steps higher than 250 kg/cm² induces an increase in $Na^+$ content. At the lower pressure of 100 kg/cm², the $Na^+$ tissue concentration appears to be lower than control values but remains higher than the $Na^+$ content measured on isolated gills incubated in the presence of oxygen at atmospheric pressure. Similarly, pressure steps of small amplitudes (100 or 250 kg/cm²) induce a decrease in $Cl^-$ content, while pressures higher than 350 kg/cm² lead to an increase in $Cl^-$ concentration. In such conditions of high hydrostatic pressure, there is also a decrease in $K^+$ concentration.

Pressure steps up to 250 kg/cm² thus appear to diminish the increase in NaCl content observed at atmosperic pressure in the experimental set-up. This observation may result either from a pressure effect on the passive $Na^+$ and $Cl^-$ entrance or from a stimulation of their active extrusion. In the last hypothesis, however, an increase in

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$K^+$ content concomitant to the decrease in $Na^+$ concentration should be expected since the $Na^+$ active transport appears to be coupled with transport of $K^+$ in the eel gills[3–13]. It thus seems that pressure acts in this experimental situation by decreasing the membrane permeability to $Na^+$ and $Cl^-$. On the other hand, the existence of a $Na^+/K^+$ coupled active transport process is in agreement with our findings that pressure steps of large amplitudes ($\geqslant 500$ kg/cm$^2$) affect in the opposite way the tissue concentrations of $Na^+$ and $K^+$. It can thus be tentatively concluded that the increase in $Na^+$ content and the decrease in $K^+$ concentration observed at high pressures are due to an inhibition of the active $Na^+$ transport system.
It is also interesting to consider that pressure acts differently on the $Na^+$ and $Cl^-$ contents. Higher pressures indeed ($p > 350$ kg/cm$^2$ for $Cl^-$ instead of 250 kg/cm$^2$ for $Na^+$) are needed to increase the $Cl^-$ content, and the magnitude of the $Cl^-$ increase measured at $p > 350$ kg/cm$^2$ is much larger than for $Na^+$ ions. This indicates that $Na^+$ and $Cl^-$ movements are governed by independant mechanisms, as already suggested by Maetz[13]. If such is the case, the large increase in tissue $Cl^-$ content observed at high pressures must be ascribed to an inhibition of the active $Cl^-$ transport process, since application of hydrostatic pressures apparently decreases the passive permeability to both $Na^+$ and $Cl^-$.
The modifications observed in transport activities may be directly or indirectly induced by high hydrostatic pressures. In the first hypothesis, pressure would act directly on the configuration of structures implicated in ion transport. That possibility has been discussed at length in another paper[9]. In the second one, pressure may, for instance, modify the disposability of oxygen to the tissue; this would then induce modification in the transport of ions. However, an effect of pressure on the oxygen tension in the incubating saline can be neglected, since there is no gas phase in the experimental set-up we have been using. Therefore, a possible indirect effect mediated through changes in cellular oxygen disposability must implicate changes in the transport of oxygen to the intracellular medium. Up to now, there is no reason to believe that hydrostatic pressure would act more specifically on oxygen transport than on ion transport. On the other hand, direct effects of pressure on ionic permeability have already been described[8,9]. Moreover, the inhibition of the active $Na^+$ transport suggested by our results can be related to a direct effect of pressure on the activity of the ($Na^+ + K^+$) ATPase extracted from the gill epithelium. Indeed, we have been unable to demonstrate any effect of oxygen on the enzyme activity and, as shown in the table, pressure exerts an inhibitory effect which increases with the magnitude of the applied pressure step.
High hydrostatic pressures would thus directly induce, besides a decrease in permeability to $Na^+$ and $Cl^-$, an inhibition of the active processes involved in the movements of these ions. Studies on ion fluxes under high hydrostatic pressures are under investigation in this laboratory in order to assess this hypothesis.

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## Differential sensitivity of newt limb regenerates to noradrenaline, as revealed by their production of cyclic AMP

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*Summary.* Regenerates of the newt forearm were incubated with noradrenaline. This increased cyclic AMP production more in the earlier than in the later stages of regeneration.

Amphibian limb regeneration depends upon the nervous system, and recent evidence supports the view that the neurotrophic agent is chemical[3,4]. Histofluorescent observations have shown the presence of catecholamines in the nerve invading the wound and in the regenerate, and inhibition of tyrosine hydroxylase retards limb regeneration[5]. Variations in the ratio of cyclic AMP to DNA during the development of the newt regenerate were reported[6]; and other papers have pointed out a possible importance of cyclic AMP during limb, lens and liver regeneration[7–11]. The purpose of the present study was to measure cyclic AMP concentrations in isolated newt limb regenerates of 2 stages, after incubation in the presence of noradrenaline. Cyclic AMP concentrations were compared with those of control regenerates at the same stages of development but which were not exposed to the drug.
*Materials and methods.* Newts, Triturus cristatus, collected in Italy were amputated through the forearm. Later, the regenerates were carefully dissected and isolated free from all stump tissue. 6–7 regenerates of early stage (bump) or 1–2 regenerates of late stage (palette) were pooled in 0.25 ml of Ringer and preincubated at 35 °C for 20 min. After addition of theophylline (final concentration 1.5 mM), an incubation was carried out at 35 °C for 10 min, with or without noradrenaline (concentration $10^{-4}$ M, total volume 0.33 ml). Homogenizer tubes containing the regenerates, were then plunged into boiling water bath for 10 min. The tissues were then homogenized (at 4 °C) and centrifuged. The pellets were used for protein determination[12]. Duplicate samples (50 µl) of the crude supernatant were taken for the satura-

1 Acknowledgments. The authors wish to thank Miss Christiane Blank for excellent technical assistance, and Dr D. Lincoln for his kind improvement of the manuscript.
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