[Frontiers in Bioscience 5, e53-62, July 1, 2000]

CARBOXYLESTERASES MOONLIGHT IN THE MALE REPRODUCTIVE TRACT: A FUNCTIONAL SHIFT PIVOTAL FOR MALE FERTILITY

Alexander T. Mikhailov and Mario Torrado

Developmental Biology Unit, Institute of Health Sciences, University of La Coruña, La Coruña, Spain

TABLE OF CONTENTS

1. ABSTRACT

This essay addresses the carboxylesterase redundancy in the male reproductive tract seemingly conserved across phyla. Evidence is provided which suggests that carboxylesterases are recruited by the male reproductive system in certain animal groups. These provide advantageous metabolic capabilities to sperm protection, sperm maturation, and sperm use. Rather than an archival record of the available data, we seek possible answers to the central question: *Why is carboxylesterase over-expression adaptive with the functioning of the male reproductive tract with respect to male fertility*? We discuss patterns of carboxylesterase over-expression and accumulation in different compartments of the male reproductive tract. We also provide evidence of how these patterns are associated with a long sperm path to egg through different local effects. The hyper-expression of carboxylesterases can play different physiological roles depending on its localization in the male reproductive system. However, all the "acquired" functions can serve the same purpose; creating conditions which maximize the fertilizing potential of the sperm. To confirm our concept and more clearly illuminate "moonlighting" roles of carboxylesterases in the male reproductive tract, requires a more extensive comparative analysis of a variety of carboxylesterases in a larger number of species.

2. INTRODUCTION

Today, virtually no specialists express any doubt that a single gene or protein can perform multiple functions that are apparently impossible to predict merely by analyzing the corresponding sequences. The computer-aided interpretation of sequences provides no guarantee against speculative predictions as many proteins consist of domains that can play different functional roles in different cell environments. This circumstance stimulated the development of new trends in studies on the molecular mechanisms of gene functioning in various biological situations.

The resulting modern approaches, such as functional genomics (1), biochemical genomics (2), and proteomics (3), allow the analysis of expression and, in some cases, functional significance of the same gene product operating in different biological settings. It appears promising to apply these methods to interspecific comparisons aimed at revealing the spectra of conservative functions performed by a certain sequence. The data obtained in this way (provided the model objects are adequate) can be used for predicting the functional significance of this sequence in many other physiological or pathological situations. Boguski (4) correctly noted that, following this trend, we should "*….change the way in which we phrase our questions from "what is the function of this protein" to "what roles does this sequence play in one or more biological processes that are operational under these conditions?..*"

The term "moonlighting" was recently proposed to describe the specific property of multifunctional enzymes that perform their "canonical" enzymatic function in a certain cell environment and play some other role in a different environment. This change of functions depends on several factors, including the level and the site of enzyme expression (5, 6). Functions of the enzyme protein can be defined both biochemically and physiologically. The functional properties assigned to a certain enzyme protein are usually centered on what we call here "molecular functions" (i.e., enzyme activities which have been determined *in vitro* by conventional molecular/biochemical methods). However, how these and other molecular properties of the enzyme can contribute to its multiple physiological functions *in vivo* and how a change (switch)

of enzyme functions might occur in different tissue or organ environments?

Using the example of carboxylesterases recruited by the male reproductive tract (7, 8), we will try to demonstrate that such functional changes do not necessarily have a simple binary ("yes–no") mechanism. More probably, we are dealing with a physiological functional shift: when the same enzyme can perform several different functions in the organism, each of them is manifested (or prevails) in a specific cell environment to which the enzyme was recruited. This paper shortly summarizes information on the overexpression of carboxylesterases in male reproductive tissues in several phyla (mollusks, insects, rodents) (8-15) and suggests that this sex-dependent overexpression can represent an evolutionary adaptation in the male reproductive system. The overall aim of this review is to provide novel integrative hypothesis demonstrating spectrum and commonality of reproductive-associated functions of carboxylesterases recruited by the male reproductive tract.

3. CARBOXYLESTERASES: FROM CANONICAL ACTIVITIES AND PROPERTIES TO SPECIFIC FUNCTIONING IN THE MALE REPRODUCTIVE TRACT

Carboxylesterases (EC 3.1.1.1) comprise a group of enzymes from a multigene carboxyl/cholinesterase family, a branch of the alpha/beta-hydrolase fold superfamily (15). Carboxylesterases hydrolyze various compounds containing carboxylic acid esters, amide and thioester functional groups. They also catalyze the hydrolysis of short- and long-chain acyl-glycerols, transesterification reactions and fatty acid ester synthesis (13, 16-18). Although, there is conclusive evidence that carboxylesterases play an important role in biological transformation of various endo- and exogenous molecules, the natural substrates and exact biological functions of these enzymes has not yet been studied in detail. Note that carboxylesterases are involved in metabolic inhibition or activation of various therapeutic drugs, and altering their activity can have important clinical implications (18).

Carboxylesterases appear to be "mosaic" molecules containing different identifiable domains (8, 16, 18). It has been demonstrated that only a portion of an enzyme may be conserved among different carboxylesterases (14, 16, 19). Particularly, the N-terminal half, including the cholinesterase-like domain, is highly conserved among carboxylesterases described. In addition, carboxylesterases share the cholinesterase-like domain with many cell recognition molecules (15, 16). The C-terminal half, containing active-site amino acids, is more diverse, which provides the versatility of the substrate binding. The latter underpins the recruitment of carboxylesterases to additional, noncatalitic (15, 20) or even structural (16) functions. Nevertheless, the sequences required for the hydrolytic capability (responsible for the hydrolysis of endo- and exogenous compounds) at the catalytic triad of carboxylesterases, acetylcholinesterases and cholesterol esterases are highly conserved (18).

A distinctive feature of carboxylesterases is their high and selective sensitivity to organophosphorous compounds (OPCs) (18, 21, 22). Moreover, the preliminary inhibition of carboxylesterase activity strongly potentiates the toxic effect of OPCs (23). Many OPCs are widely used as pesticides or herbicides. Some of these agents have a selective damaging effect on the male reproductive tract, which eventually manifests itself in disturbances of sperm cell differentiation and maturation (24, 25). Carboxylesterases participate in the process of specific OPC detoxification by: (1) binding OPCs to their active centers and, thus, sequestering them in the cell or (2) hydrolyzing the ester bonds of OPC molecules (26, 27). These properties of carboxylesterases provided a primarily basis for the development of a concerning the functions of these enzymes in the male reproductive tract.

The idea that carboxylesterases can contribute to the maintenance of male reproductive health appeared when we compared apparently unrelated sets of data obtained by different research teams (including ours) in independent experiments.

On the one hand, the hyper-expression of carboxylesterases in the male reproductive tract proved to be characteristic of animals belonging to the taxa of different phyla, namely, bivalve mollusks (9-11), fruit flies (12, 20, 28), and rodents (13, 29). On the other, there was evidence for the structural, biochemical, and immunochemical similarity of these carboxylesterases among the species studied (8, 10, 12, 14, 20, 30). In aggregate, these data suggested that, in the course of evolution, the carboxylesterase genes were recruited for specific functioning in the male reproductive tract (7, 8). In the taxa of each phylum studied (i.e., among bivalves, fruit flies, and mammals), the phenomenon of carboxylesterase hyper-expression in the male reproductive tract is characteristic of some animals but is not universal (12, 15, 20, 28). This fact provided additional evidence for the hypothesis of possible specific recruitment of these enzymes by the male reproductive system.

It is the aforementioned phenomenon that provided a logical connecting link to the following interesting observations. It has long been discovered that some insect taxa (particularly, mosquitoes) rapidly become resistant insecticides, including OPCs. The basic mechanism responsible for the development of OPC insecticide resistance proved to be associated with the hyper-expression of carboxylesterases in the insect organism (31, 32). Contrarily, in houseflies and sheep blowflies carboxylesterase-mediated resistance to OPCs involves changed enzyme substrate specificity (as a result of amino acid substitutions). This reduces canonical esterase activity, on the one hand, and sharply enhances a capacity for hydrolyzing OPCs, with no evidence for abundance of enzymes (33, 34). It is interesting that some carboxylesterases expressed in the *Drosophila* male reproductive tract (for example, Esterase S; see Fig. 1) have no "true" esterase activity (15, 20, 30). This suggests that they can fulfill sperm protective and other functions in the male reproductive system.

Table 1. Over-expression and accumulation of male-dependent carboxylesterases in different compartments of the reproductive tract

Compartment	Carboxylesterase	Ref.
Testicular	Mussel MAP	9
parenchyma	Rat hydrolase A	13
	Mouse Esterase 6	29
Excurrent	Mussel MAP	11
(post-testicular)	Scallop MAP	7
duct system	Fruit fly Esterase 6	56
	Mouse Esterase 28	81
Efferent/ejaculatory	Mussel MAP	39
duct system	Fruit fly Esterase 6	28
	Fruit fly Esterase S	12

MAP: male associated polypeptide of bivalve mollusks

4. MALE REPRODUCTIVE-TRACT CARBOXYLESTERASE OVER-EXPRESSION IS ESSENTIAL FOR MALE FERTILITY?

Our concept represents a series of deductions leading to the basic conclusion that carboxylesterases recruited by the male reproductive tract are a physiological component contributing to the male reproductive health. The recruitment of these enzymes is not accompanied by the loss of their essential properties, including the detoxifying activity. The hyper-expression of carboxylesterases can play different physiological roles depending on its localization in the male reproductive system. Nevertheless, all the "acquired" functions may serve the same purpose: to create conditions for normal sperm cell production, sperm release and sperm use. This is why we call attention to the carboxylesterase physiological shift that appears to be essential for the male reproductive health. We also attempt to incorporate such functional adaptation with the possible role of carboxylesterases in protecting the male reproductive tract against various xenobiotics (8).

As noted above, studies on the animals characterized by radically different reproductive strategies and patterns (bivalves, fruit flies, and rodents) revealed a general trend in carboxylesterase hyper-expression in the male reproductive system (Figure 1, Table 1). Considerable amounts of male-specific carboxylesterases proved to accumulate in different compartments of this system, namely, (1) epithelial seminiferous tubules and the adjacent interstitial tissue; (2) the epithelium of the excurrent (post-testicular) and efferent duct system, and (3) luminal fluids of the efferent ducts, including seminal plasma. In addition, carboxylesterases are always present in both extracellular and cell-bound fractions of semen in bivalve mollusks (10), sea urchins (35), fruit flies (12, 28, 36) and men (37-39).

In other words, carboxylesterases in the male reproductive tract seem to be an essential component of the male-specific microenvironment throughout the long developmental pathway from a sperm stem cell to a mature fertile spermatozoon. Moreover, they have effect on the fate of spermatozoa in the female organism (28). Structural compartments of the male reproductive system (testis, epididymis, ducts, etc.) are known to provide for different stages of sperm cell differentiation, maturation, storage, and motility development (40-43). Hence, it appeared logical to assume that carboxylesterase over-expression and accumulation found in each of these compartments have different functional roles depending on the stage of the sperm cell pathway at which they can exert their conditioning influence. As will be shown below, this assumption proved to be valid.

4.1. Testis-associated carboxylesterases

In the testis, the functional role of carboxylesterases is apparently determined by their involvement in testosterone biosynthesis and the protection of Leydig cells from the effect of damaging and toxic agents. In rats, typical "testis-specific" toxic substances, such as ethane dimethane sulfonate (alkylating antitumor agent) (13, 44, 45), tri-*o*-cresyl phosphate (plasticizer) (46), and molinate (herbicide) (47, 48), inhibit the activity and expression of testicular carboxylesterase (hydrolase A) and significantly reduce testicular and plasma testosterone concentrations. Morphologically, their effect is manifested in structural changes of Leydig and Sertoli cells and signs of inhibited spermatogenesis.

It is noteworthy that phenylmethylsulfonyl fluoride, a serine protease inhibitor that also inactivated various carboxylesterases (44), inflicts similar testicular damage. There is evidence that testicular carboxylesterases can possess cholesterol esterase activity, which is necessary for the release of cholesterol at the first stage of androgen synthesis (48). Hence, their inhibition can disturb the synthesis of testosterone by Leydig cells, and this, in turn, can have a pleiotropic adverse effect on the male reproductive system. In addition, molinate reduces sperm fertilizing potential concurrently with reductions in sperm motility (49).

Experiments showed that carboxylesterases are capable of high-affinity binding to active metabolites of molinate (48) and tri-*o*-cresyl phosphate (46) and, hence, can temporarily protect Leydig cells from their toxic action (13). On this basis, we suppose that the hyper-accumulation of these enzymes in Leydig cells (as well as in the sperm, see below) is a physiological buffering mechanism preventing the development of testicular injury under the effect of certain environmental factors, including man-made pollutants (8). An especially important fact in this context is that some agents toxic for testicles are used on an industrial scale, e.g., as plasticizers in lacquers (tri-*o*-cresyl phosphate) (46) and herbicides (molinate) (47).

Studies on various models demonstrated that, in addition to testes, male-specific carboxylesterases are hyperexpressed in the epididymis, the epithelium of ejaculatory bulb glands, seminal vesicles, and ejaculatory ducts (see Figure 1, Table 1). Hence, by analogy with the data discussed above, we suppose that carboxylesterase hyper-production in these structures of the male reproductive tract also serves as a protective mechanism against toxic influences. In other words, *one probable function of carboxylesterases is to create an integral circuit*

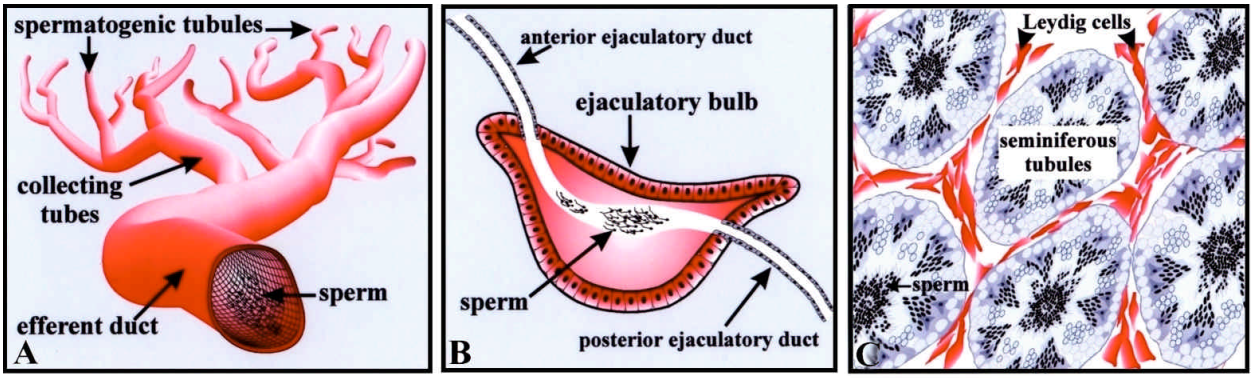

Figure 1. Patterns of sex-dependent carboxylesterase over-expression (red) in the male reproductive tract of bivalve mollusks (A), fruit flies (B), and rodents (C). Schematic morphology of the corresponding reproductive-tract tissues/organs is also shown. **A:** general view of the *Mytilus galloprovincialis* tubular gonad, showing the graded distribution of "male-associated polypeptide" (MAP, a carboxylesterase-like protein) with peak levels in the epithelium of efferent ducts and relatively lower levels in the epithelium of spermatogenic tubules (11, 14). In bivalves, sperm differentiation proceeds in follicle-like seminiferous tubules; the sperm cells undergo maturational changes along the efferent duct system. The gonad ducts serve as sperm storage organs and as a transport route for spermatozoa. **B**: sagital-section view of the *Drosophila virilis* ejaculatory bulb, indicating epithelial cells over-expressing Esterase S (12). In contrast of the mammalian situation, in which seminal fluid is mainly a secretion of the seminal vesicles, insect seminal fluid may be derived from any or all the glands of the male reproductive tract. **C**: cross-section view of the *Rattus norvegicus* testis, showing Leydig cells over-expressing hydrolase A (13).

(like a "protective shell") in the male reproductive tract and protect its different parts and sperm cells against xenobiotics by binding them rather than metabolizing. Note that the number of enzyme molecules seems to play a crucial role in the carboxylesterase-mediated detoxification potential of a tissue (21).

The suggestion about the caboxylesterase-mediated protective circuit in the male reproductive tract mentions only man-made pollutants as possible targets. Note that the testicular toxic chemicals under consideration are only examples which indicate to a possible link between carboxylesterase hyper-expression and the protection of male reproductive-tract tissues.

It is likely that the use of male reproductive toxicants, such as pesticides, xenobiotic phthalates and other man-made compounds (8), could lead to selection of the mutations resulting in carboxylesterase hyper-expresion in the male reproductive tract, but only in insects, which evolve rapidly. The problem with this interpretation is to understand why carboxylesterase over-expression is also observed in the male reproductive-tract tissues of other phyla (i.e., bivalve mollusks, rodents). A possible explanation could be that carboxylesterases have been recruited to the male reproductive tract from detoxifying enzymes in the course of evolution (8). The recruitment was not accompanied by the complete loss of their "ancestral" detoxifying activity. At the same time, carboxylesterase hyper-expression in the male reproductive tract became physiologically associated with processes of sperm maturation and sperm release rather than with the acquisition of protective properties by reproductive tissues. The available information, which will be discussed below, permits this scenario to be sketched that may reflect reality.

4.2. Luminal and seminal-plasma carboxylesterases

Studies on bivalves (11), fruit flies (28), mice (50) and men (37, 51, 52) demonstrated that carboxylesterases synthesized in the male reproductive tract are not only retained in tissues but are also released and accumulated in the corresponding luminal fluids and seminal plasma, where they can reach considerably high concentrations. This fact suggested that carboxylesterases, along with other male-dependent proteins and enzymes, are involved in creating a specific luminal environment (53) in which spermatozoa formed in testes acquire motility and fertilizing ability. Note that terminally differentiated spermatozoa are still immature and incapable of effective motility upon their release from the seminiferous epithelium. The capacity to move develops when spermatozoa pass through the post-testicular duct system (40, 54).

In the male gonad duct system of the mussel *Mytilus galloprovincialis*, the mature spermatozoa are retained within a transparent, gel-like luminal structures (the so-called sperm morulae). Sperm cells must be released from these structures before passing through the collecting tubes and being ejected from the gonophores (Mikhailov, Torrado, unpublished). We believe that sperm activation in mussels is accomplished in two stages. The first results in the liberation of mature spermatozoa from the gel-like storage structures so that they can freely swim in the lumina of collecting and ejaculatory ducts. The second stage involves the subsequent development of

sperm motility, which takes place when spermatozoa are ejaculated and exposed to sea water.

It appears that luminal fluid proteins stimulate the first stage. An exceptionally high concentration of the so-called *male associated polypeptide* (MAP; a carboxylesterase-like protein) in the *M. galloprovincialis* seminal fluid and semen suggests its involvement in the process of sperm liberation occurring in the efferent gonad duct compartment before spawning is activated (10, 11).

How valid is such a suggestion? In *Drosophila melanogaster*, male-predominant Esterase 6 (biochemically similar to mussel MAP) (8) is highly expressed in the anterior ejaculatory duct and is partially retained in the male reproductive-tract lumen after copulation. It was proposed that the enzyme can degrade seminal lipids, thereby reducing the viscosity of stored semen (55). This assumption was especially interesting in view of the observations on Esterase 6 activity in the *D. melanogaster* accessory glands (56, 57), which, along with the anterior ejaculatory duct, are secretory organs contributing to the protein content of the seminal fluid.

Esterase 6 of the male accessory glands also displays a low triacylglycerol-hydrolyzing activity. This activity is higher in wild-type flies than in Esterase 6 null mutants (57), which also lack any detectable carboxylesterase activity and, as a consequence, are characterized by reduced sperm motility and sperm use (see below). Phenotypically, Esterase 6-null males are similar to reproductively immature wild-type males, as they inseminate females with smaller amounts of sperm released at slower rates, compared to those in matings with mature wild-type males (55). Moreover, studies on different *D. melanogaster* lines revealed strong positive correlations between the level of male Esterase 6 activity and reproductive fitness (58, 59). In the virilis group, low Esterase S amounts were detected in the ejaculatory bulb and seminal fluid of sterile males (M. Ludwig, personal communication).

Studies on the enzyme profiles of human seminal plasma and spermatozoa allowed the detection of carboxylesterases that could be potentially involved in sperm metabolism and in the process of fertilization. However, no significant differences in the esterase spectra of seminal plasma between normal, fertile donors and subfertile or azoospermic patients were revealed (37). Moreover, attempts to demonstrate any significant correlation between the esterase enzyme pattern and the percentage of motile cells in ejaculated sperm were unsuccessful (51).

The data discussed above suggest that carboxylesterases of seminal fluids do not contribute directly to the motility of spermatozoa proper. *More likely, they are involved in the processes ensuring that mature sperm stored in the male reproductive tract is released in proper doses shortly before or during copulation.* Note that level of sperm release is one of the factors that can cause sperm limitation, especially in free-spawning organisms (60).

4.3. Sperm cell carboxylesterases

The esterase equipment of spermatozoa is closely similar to that of seminal plasma, suggesting that most of sperm-associated carboxylesterase activity is accounted for by the male reproductive-tract secretions. There is substantial evidence that seminal-plasma-derived carboxylesterases can promote the release of spermatozoa in the female reproductive tract, and, hence, the increase in their motility and use, although the specific mechanisms of this effect are as yet unknown. The corresponding data were obtained in studies on the release of stored spermatozoa from the spermatheca into the *D. melanogaster* female reproductive tract. Initially, it was noted that this process is stimulated by male-derived Esterase 6 present in the ejaculate. Experiments on crossing females with males differing in the level of Esterase 6 activity demonstrated that the rate of sperm release from the female spermatheca depends on the level of Esterase 6 in semen (55). The latter suggests a possible role of seminal Esterase 6 in sperm competition, mediated by incapacitation or inefficient use of resident sperm.

In bivalve mollusks, different carboxylesterase forms (mussel MAP and *D. virilis* Esterase S-like protein) were detected not only in the cell-free fraction but also in the sperm-containing fraction of the ejaculate. Extensive and prolonged washing of the sperm suspension did not cause a detectable reduction of the corresponding positive immuno-signals of spermatozoa (10, 11). This indicates that in bivalves, carboxylesterases might be tingly associated with the spawned sperm cells. Therefore it could be expected that they are implicated in processes of sperm capacitation (14).

The amplified carboxylesterase genes are also constitutively present in the semen of *Culex pipens* mosquitoes (61). However, the role of these enzymes in sperm metabolism is as yet unclear.

Studies on the model of mammalian spermatozoa washed to remove seminal plasma showed that carboxylesterases are an essential component of both spermatozoon plasmalemma (62) and the acrosomal cap (63-65). The acrosome is a large secretory vesicle in the apical region of the sperm head. Only the normal acrosome reaction allows spermatozoa to penetrate the zona pellucida and fuse with the oocyte plasma membrane.

Mammalian sperm do not respond to inducers of the acrosome reaction immediately after ejaculation. In the female reproductive tract, mammalian sperm undergo a process called capacitation, which results in alteration of sperm motility and acrosome reaction (66). An essential feature of capacitation is the removal of cholesterol from the acrosomal membrane of sperm (67, 68). Note that cholesterol seems to be the major seminal-plasma inhibitor which prevents human sperm from becoming acrosomally responsive (69, 70). Mammalian carboxylesterases hydrolyze numerous esterified lipids, including cholesterol esters (18, 71). Therefore, it would seem reasonable to suggest that *sperm-associated carboxylesterases can contribute to capacitation processes through their ability to remove cholesterol from the sperm plasma membrane.*

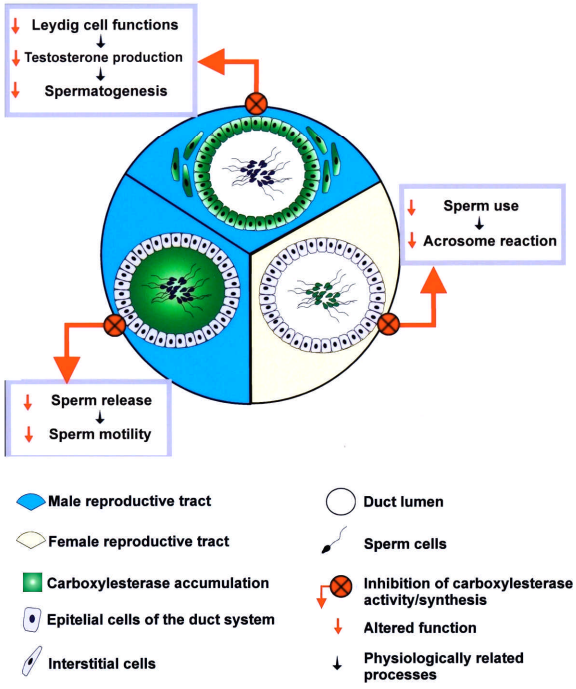

Figure 2. A schematic diagram of carboxylesterase functional linkages with sperm cell pathway. Depending on the stage of sperm cell pathway, carboxylesterase overexpression/accumulation in the male reproductive tract can contribute to spermatogenesis, sperm release, and further modifications of the sperm plasma membrane. Inhibition of carboxylesterase activity/expression correlates with alterations in: (1) testosterone metabolism and spermatogenesis (47, 48); (2) liberation of mature sperm from storage structures (55); (3) semen liquefaction and sperm use (28, 55, 58), and (4) sperm capacitation (the latter remains to be elucidated; see text for further details).

Bradford *et al.* (72) advanced the hypothesis that sperm carboxylesterases in mammals are functionally similar to the so-called corona penetrating enzyme and, hence, can condition the sperm–egg interaction. However, the experimental evidence for this possibility remains controversial (62) suggesting an alternative interpretation of the results obtained.

The carboxylesterase ancestral gene already existed before the divergence of invertebrates and vertebrates (15, 19, 73). It appears that carboxylesterase genes have been recruited by the male reproductive tract to function in additional, non-detoxification processes, such as testosterone metabolism, liquefaction of the seminal fluid, sperm cell displacements, and modifications of the plasma membrane of mature spermatozoa (Figure 2). Reproductive-tract-associated functions of individual carboxylesterase proteins as summarized above appear to be more compatible on the whole with their role in the maintenance of sperm fertilizing potential rather than with their "ancestral" detoxification functions. At the genome level, this functional shift could be interpreted in several ways – depending on whether we prefer gene duplication mechanisms or changing the control of expression of the genes (6, 8, 73-77). It is evident that male reproductive-tract proteins (including several carboxylesterases) undergo a strong selection that may favor altered gene expression if it is adaptive for reproduction (78, 79). Note that a successful reproduction requires the close matching of male and female reproductive-tract protein traits (80). Therefore, the most interesting aspect is how carboxylesterase activities became adapted during evolution to accomplish new physiological functions with a reproductive outcome in species with quite different reproductive patterns and sexuality.

5. PERSPECTIVES

The problem of male reproductive health and conditions of its maintenance has been of interest to people since ancient times. Even some biblical texts represent medical treatises discussing the relationship between the testis (male gonad), on the one hand, and male fertility and the procreative functions of populations, on the other. In this sense, the concept described above appears relevant, as it concerns the possible involvement of carboxylesterases in processes providing for the male reproductive health. The precise functional roles of carboxylesterases "moonlighting" in the male reproductive tract are poorly known as yet. Nevertheless, the fact that their hyper-expression in this system is equally characteristic of mollusks, insects, and mammals provides evidence that the possible male-associated functions of these enzymes are fairly conservative. This, in turn, opens up opportunities for the comparative analysis of their reproduction-related functions in various model organisms. Technologies and procedures applicable to such an analysis can range from the use of loss-of-function mutations to experiments on carboxylesterase inhibition and activation in co-cultivated gonad somatic cells and spermatozoa.

The application of these techniques to the study of reproductive-tract carboxylesterases is going to provide many interesting data (8). To take full advantage of these advances we should try to use model organisms (bivalve mollusks, fruit flies) (8, 14) where hypothesis can be tested and develop experimental approaches in organisms (mammals) where such trends are not yet available. This will be the way to get clear understanding of the molecular and physiological mechanisms by which carboxylesterases contribute to the male reproductive health. By understanding the underlying mechanisms, it may be possible to develop new techniques to yield information regarding the prognosis of male subfertility. Particularly, we hope that biochemical testing of carboxylesterase expression in the semen could be used to detect the corresponding subtle changes that may affect the fertilizing potential of the sperm.

6. ACKNOWLEDGEMENT

We thank Professor Scott Gilbert for his comments on a preliminary version of the manuscript and Doctor Mikhail Ludwig for communicating his unpublished observations on *Drosophila* sterile males. We also acknowledge Professor Joram Piatigorsky and Professor

John Gurdon for useful discussions prior to the writing of the paper. We also wish to extend our gratitude to Mr. S. Tabibzadeh, Editor of "Frontiers in Bioscience", for his attention and comments, and to Doctor Nikolayi Gorgolyuk for English language improvements as well. Several well-known observations and concepts are stated without primary references, which are included in the reviews cited. We apologize to our colleagues in the field whose more primary publications were not cited. Work on male reproductive-tract carboxylesterases of bivalves, fruit flies and mammals in the Developmental Biology Unit of A.T.M. is funded by the Galicia Government Grant, 16101A 98. M.T. is supported by a postdoctoral fellowship from the Institute of Health Sciences (University of La Coruña, Spain).

7. REFERENCES

1. Rastan S. & L.J. Beeley: Functional genomics: owing forwards from the databases. *Curr Biol* 7, 777-783 (1997)

2. Martzen M.R, S.M. McCraith, S.L. Spinelli, F.M. Torres, S. Fields, E.J. Grayhack & E.M. Phizicky: A biochemical genomics approach for identifying genes by the activity of their products. *Science* 286, 1153-1155 (1999)

3. Anderson N.L. & N.G. Anderson: Proteome and proteomics: New technologies, new concepts, and new words. *Electrophoresis* 1853-1861 (1998)

4. Boguski M.S.: Biosequence exegesis. *Science* 286, 453-455 (1999)

5. Jeffery C.J.: Moonlighting proteins. *Trends Biochem Sci* 24, 8-11 (1999)

6. Piatigorsky J.: Gene sharing in lens and cornea: facts and implications. *Retinal Eye Res* 17, 145-174 (1998)

7. Mikhailov A.T, M. Torrado, M. Paz & L.I. Korochkin: Gonad recruitment of carboxylesterase genes during evolution of the reproductive system: conserved male-specific over-expression in mussels, fruitflies, and mammals. In: Molecular strategies in biological evolution. Ed. Caporale LH, Ann N Y Acad Sci 870, 389-393 (1999)

8. Mikhailov A.T, & M. Torrado: Carboxylesterase overexpression in the male reproductive tract: a universal safeguarding mechanism? *Reprod Fertil Dev* 11, 133-145 (1999).

9. Mikhailov A.T, M. Torrado & J. Méndez: Sexual differentiation of reproductive tissue in bivalve molluscs: identification of male associated polypeptide in the mantle of the *Mytilus galloprovincialis*. *Int J Dev Biol* 39, 545-548 (1995)

10. Mikhailov A.T, M. Torrado, L.I. Korochkin, M.A. Kopantzeva & J. Méndez: Male-predominant carboxylesterase expression in the reproductive system of molluscs and insects: immunochemical and biochemical similarity between *Mytilus* male associated polypeptide (MAP) and *Drosophila* sex-specific esterase S. *Comp Biochem Physiol* 117B,197-208 (1997)

11. Torrado M. & A.T. Mikhailov: Male-associated polypeptide (MAP) expression in different compartments of the reproductive system of the mussel, *Mytilus galloprovincialis*: immunocytochemical and Western blot study. *Cell Tissue Res* 292, 165-178 (1998)

12. Korochkin L, M. Ludwig, N. Tamarina, I. Uspensky, G. Yenikolopov, R. Khechumijan, M. Kopantzeva, M. Evgeniev, B. Kuzin, T. Bakayeva, L. Mndjoian, O. Malevantschuk, V. Tsatrian, A. Ivanov & S. Lukianov: Molecular genetic mechanisms of tissue-specific esterase isozyme and protein expression in *Drosophila*. In: Isozymes: structure, function, and use in biology and medicine. Eds: Market C, Scandalios J, Alan R. Liss, NY 399-440 (1990)

13. Yan B, D. Yang, M. Brady & A. Parkinson: Rat testicular carboxylesterase: cloning, cellular localization and relationship to liver hydrolase A. *Arch Biochem Biophys* 316, 899-908 (1995)

14. Torrado M, A. Mikhailov, M. Paz & L. Korochkin: Comparative analysis of male reproductive-tract carboxylesterases: sex-dependent expression is shared by species with different patterns of reproduction and sexuality. In: Recent developments in comparative biochemistry and physiology. Ed: Pandalai SG, Transword Res Network (2000) (in press)

15. Oakeshott J.G, C. Claudianos, R.J. Russel & G.C. Robin: Carboxyl/cholinesterases: a case study of the evolution of a successful multigene family. *BioEssays* 21, 1031-1042 (1999)

16. Yan B, L. Matoney & D. Yang: Human carboxylesterases in term placentae: enzymatic characterization, molecular cloning and evidence for the existence of multiple forms. *Placenta* 20, 599-607 (1999)

17. Kaphalina B.S, R.R. Fritz & G.A.S. Ansari: Purification and characterization of rat liver microsomal fatty acid ethyl and 2-chloroethyl ester synthetase and their relationship to carboxylesterase (p*I* 6.1). *Chem Res Toxicol* 10,211-218 (1997)

18. Satoh T. & M. Hosokawa: The mammalian carboxylesterases: from molecules to functions. *Annu Rev Pharmacol Toxicol* 38, 257-288 (1998)

19. Takagi Y, T. Omura & M. Go: Evolutionary origin of thyroglobulin by duplication of esterase genes. *FEBS Lett* 282, 17-22 (1991)

20. Oakeshott J.G, T.M. Boyce, R.J. Russell & M.J. Healy: Molecular insights into the evolution of an enzyme; esterase 6 in *Drosophila*. *Trends Ecol Evol* 10, 103-110 (1995)

21. Chanda S.M, S.R. Mortensen, V.C. Moser & S. Padilla: Tissue-specific effects of chlorpyrifos on carboxylesterase and cholinesterase activity in adults rats: an *in vitro* and *in vivo* comparison. *Fundam Appl Toxicol* 38,148-157 (1997)

22. Moser V.C, S.M. Chanda, S.R. Mortensen & S. Padilla: Age- and gender-related differences in sensitivity to chlorpyrifos in the rat reflect developmental profiles of esterase activities. *Toxicol Sci* 46, 211-222 (1998)

23. Shih D.M, L. Gu, Y.R. Xia, M. Navab, W.F. Li, S. Hama, L.W. Castellani, C.E. Furlong, L.G. Costa, A.M. Fogelman & A.J. Lusis: Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature* 394, 284-287 (1998)

24. Yousef M.I, M.H. Salem, H.Z. Ibrahim, S. Helmi, M.A. Seehy & K. Bertheussen: Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits. *J Environ Sci Health* 30, 513-534 (1995)

25. Akbarsha M.A. & P. Sivaamy: Male reproductive toxicity of phosphamidon: physiological changes in epididymis. *Indian J Exp Biol* 36, 34-38 (1998)

26. Jokanovic M, M. Kosanovic & M. Maksimovic: Interaction of organophosphorus compounds with carboxylesterases in the rat. *Arch Toxicol* 70, 444-450 (1996)

27. Tang J. & J.E. Chambers: Detoxification of paraoxon by rat liver homogenate and serum carboxylesterases and A-esterases. *J Biochem Mol Toxicol* 13, 261-268 (1999)

28. Richmond R.S, K.M. Nielson, J.P. Brady & E.M. Snella: Physiology, biochemistry and molecular biology of the *Est-6* locus in *Drosophila melanogaster*. In: Ecological and evolutionary genetics of *Drosophila*. Eds: Barker JSF, Stamer WT, MacIntyre RJ, Plenum Press, NY 273-292 (1990)

29. Von Deimling O, A. Ronai & S. de Looze: Non-specific esterases of mammalian testis. Comparative studies on the mouse (*Mus musculus*) and rat (*Rattus norvegicus*). *Histochem* 82, 547-555 (1985)

30. Tamarina N.A, M.Z. Ludwig, G.N. Enikolopov, B.A. Kuzin & LI. Korochkin: Analysis of the protein product of the tissue-specific gene *Est-S* in *Drosophila virilis*. *Proc (Doklady) Russ Acad Sci* 316, 228-230 (1991)

31. Guillemaud T, N. Makate, M. Raymond, B. Hirst & A. Callaghan: Esterase gene amplification in *Culex pipiens*. *Insect Mol Biol* 6, 319-327 (1997)

32. Hemingway J. & S.H. Karunaratne: Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med Vet Entomol* 12, 1-12 (1998)

33. Newcomb R.D, P.M. Campbell, D.L. Ollis, E. Cheah, R.J. Russell & J.G. Oakeshott: A single amino acid substitution convert a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc Natl Acad Sci USA* 94, 7464-7468 (1997)

34. Claudianos C, R.J. Russell & J.C. Oakeshott: The same amino acid substitution in orthologous esterases confers organophosphate resistance on the house fly and a blowfly. *Insect Biochem Mol Biol* 29, 675-686 (1997)

35. Resing K, J.D. Green & K.A. Walsh: A 53,000-Da esterase in *Strongylocentrotus purpuratus* semen is derived from phagocyte cells, not sperm. *Dev Biol* 107,87-93 (1985)

36. Meikle DB, K.B. Sheehan, D.M. Phillis & R.C. Richmond: Localization and longevity of seminal-fluid esterase 6 in mated female *Drosophila melanogaster*. *J Insect Physiol* 36, 93-101 (1990)

37. Guerin J.F, Y. Menezo & J.C. Czyba: Enzyme comparative study of spermatozoa and seminal plasma in normal and subfertile man. *Arch Androl* 3, 251-257 (1979)

38. Taniguchi K, K. Mamba & M. Kitahama: On localization of enzymes in human spermatozoa. III. Esterase and acrosomal proteinase. *Nippon Hoigaku Zasshi* 40, 24-29 (1986)

39. Torrado M, M. Paz & A.T. Mikhailov: Seminal fluid carboxylesterases: bivalve mollusks like fruit flies and humans. Proc. 7th Spanish Congr. Aquaculture. Las Palmas de Gran Canaria, (2000) (in press)

40. Hegde U.C.: Epididymal sperm maturation proteins. *Indian J Biochem Biophys* 33, 103-110 (1996)

41. Berruti G.: Signaling events during male germ cell differentiation: bases and perspectives. *Front Biosci* 3, 1097-1108 (1998)

42. Cooper T.G.: Role of the epididymis in mediating changes in the male gamete during maturation. In: Tissue renin-angiotensin systems. Eds: Mukhopadhyay AK, Raizada MK, Plenum Press, NY 87-101 (1995)

43. Moore H.D.: Contribution of epididymal factors to sperm maturation and storage. *Andrologia* 30, 233-239 (1998)

44. Morgan E.W, B. Yan, D. Greenway, D.R. Petersen & A. Parkinson: Purification and characterization of two rat liver microsomal carboxylesterases (hydrolase A and B). *Arch Biochem Biophys* 315, 495-512 (1994)

45. Hess R.A.: Effects of environmental toxicants on the efferent ducts, epididymis and fertility. *J Reprod Fertil* 53(Suppl.), 247-259 (1999)

46. Chapin R.E, J.L. Phelps, S.J. Somkuti, J.J. Heidel & L.T. Burka: The interaction of Sertoli and Leydig cells in the testicular toxicity of tri-*o*-cresyl phosphate. *Toxicol Appl Pharmacol* 104, 483-495 (1990)

47. Ellis M.K, A.G. Richardson, J.R. Foster, F.M. Smith, P.S. Widdowson, M.J. Farnworth, R.B. Moor, M.R. Pitts & G.A. Wickramaratne: The reproductive toxicity of molinate and metabolites to the male rat: effects on testosterone and sperm morphology. *Toxicol Appl Pharmacol* 151, 22-32 (1998)

48. Jewell W.T. & M.G. Miller: Identification of a carboxylesterase as a the major protein bound by molinate. *Toxicol Appl Pharmacol* 149, 226-234 (1998)

49. Berger T, M.G. Miller & C.M. Horner: *In vitro* fertilzation after *in vivo* treatment of rats with three reproductive toxicants. *Reprod Toxicol* 14, 45-53 (2000)

50. Abou-Haila A. & M.A. Fain-Maurel: Selective action of androgens on the molecular forms of esterases characterized by two-dimensional electrophoresis in the epididymis and vas deferens of the mouse. *Int J Androl* 14, 201-222 (1991)

51. Prasad R, D. Mumford & H. Gordon: Lactate and malate dehydrogenase and alpha-esterases in oligospermia. *Fertil Steril* 27, 832-835 (1976)

52. Roberts T.K, P.L. Masson, R. Lauwerys & J.F. Heremans: Two esterases common to plasma seminal, other external secretions and leukocytes in man. *Andrologia* 8, 67-71 (1976)

53. Kirchhoff C.: Molecular characterization of epididymal proteins. *Rev Reprod* 3, 86-95 (1998)

54. Jones R.C.: To store or mature spermatozoa? The primary role of the epididymis. *Int J Androl* 22, 57-67 (1999)

55. Gilbert D.G.: Ejaculated esterase 6 and initial sperm use by female *Drosophila melanogaster*. *J Insect Physiol* 27, 641-650 (1981)

56. Sheehan K, R.C. Richmond & B.J. Cochrane: Studies of esterase 6 in the *Drosophila melanogaster*. III. The development patterns and tissue distribution. *Insect Biochem* 9, 443-450 (1979)

57. Smith G.M, K. Rothwell, S.L. Wood, S.J. Yeaman & M. Bownes: Specificity and localization of lipolytic activity in adult *Drosophila melanogaster*. *Biochem J* 304, 775-779 (1994)

58. Richmond R.C, D.G. Gilbert & K.B. Sheehan: Esterase 6 and reproduction in *Drosophila melanogaster*. *Science* 207, 1483-1485 (1980)

59. Saad M, A.Y. Game, M.J. Healy & J.G. Oakeshott: Associations of esterase 6 allozyme and activity variation with reproductive fitness in *Drosophila melanogaster*. *Genetica* 94, 43-56 (1994)

60. Yund P.O.: How severe is sperm limitation in natural populations of marine free-spawners? *Trend Ecol Evol* 15, 10-13 (2000)

61. Raymond M. & N. Pasteur.: The amplification of B1 esterase gene in the mosquito *Culex pipiens* is present in gametes. *Nucleis Acid Res* 17, 711-712 (1989)

62. Fain-Maurel M.A. & A. Abou-Haila: Subcellular distribution of the nonspecific esterase in the mouse epididymis with special reference to regional differences. *Anat Rec* 214, 148-153 (1986)

63. Meizel S, D. Boggs & J. Cotham: Electrophoretic studies of esterases of bull spermatozoa, cytoplasmic droplets and seminal plasma. *J Histochem Cytochem* 19, 226-231 (1971)

64. Bryan J.H.D. & R.R. Unnithan: Non-specific esterase activity in bovine acrosomes. *Histochem J* 4, 413-419 (1972)

65. Kakar S.S. & S.R. Anand: Acrosomal damage and enzyme leakage during freeze preservation of buffalo spermatozoa. *Indian J Exp Biol* 22, 5-10 (1984)

66. Topper E.K, G.J. Killian, A. Way, B. Engel & H. Woelders: Influence of capacitation and fluids from the male and female genital tract on the zona binding ability of bull spermatozoa. *J Reprod Fertil* 115, 175-183 (1999)

67. Martinez P. & A. Morros: Membrane lipid dynamics during human sperm capacitation. *Front Biosci* 1, 103-117 (1996)

68. Visconti P.E, H. Galantino-Homer, X. Ning, G.D. Moore, J.P. Valenzuela, C.J. Jorgez, J.G. Alvarez & G.S. Kopf: Cholesterol efflux-mediated signal transduction in mammalian sperm: beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *J Biol Chem* 274, 3235-3242 (1999)

69. Cross N.L.: Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. *Biol Reprod* 54, 138-145 (1996)

70. Cross N.L.: Effect of methyl-beta-cyclodextrin on the acrosomal responsiveness of human sperm. *Mol Reprod Dev* 53, 92-98 (1999)

71. Hosokawa M, T. Maki & T. Satoh: Characterization of molecular species of liver microsomal carboxylesterases of several animal species and humans. *Arch Biochem Biophys* 277, 219-227 (1990)

72. Bradford M.M, R.A. McRorie & W.L. Williams: Involvement of esterases in sperm penetration of the corona radiata of the ovum. *Biol Reprod* 15, 102-106 (1976)

73. Oakeshott J.G, E.A. van Papenrecht, T.M. Boyce, M.J. Healy & R.J. Russell: Evolutionary genetics of *Drosophila* esterases. *Genetica* 90, 239-268 (1993)

74. Ganfornina M.D. & D. Sánchez: Generation of evolutionary novelty by functional shift. *BioEssays* 21, 432-439 (1999)

75. Todd A.E, C.A. Orengo & J.M. Thornton: Evolution of protein function from a structural perspective. *Curr Opin Chem Biol* 3, 548-556 (1999)

76. Hughes A.L.: The evolution of functionally novel proteins after gene duplication. *Proc R Soc Lond B Biol Sci* 256, 119-124 (1994)

77. Wagner A.: The fate of duplicated genes: loss or new function? *BioEssays* 20, 785-788 (1998)

78. Miyta T, I. Kuma, N. Iwabe & N. Nikon: A possible link between molecular evolution and tissue evolution

demonstrated by tissue-specific genes. *Jpn J Genet* 69, 473-480 (1994)

79. Chen P.S.: The accessory gland proteins in male *Drosophila*: structural, reproductive, and evolutionary aspects. *Experientia* 52, 503-510 (1996)

80. Gavrilets S.: Rapid evolution of reproductive barriers driven by sexual conflict. *Nature* 403, 886-889 (2000)

81. Von Deimling O. & B. Wassmer: Genetic identification of non-specific esterases of the mouse cauda epididymis and description of esterase-28, a new carboxylesterase isoenzyme (EC 3.1.1.1). *J Reprod Fert* 88,41-50 (1990)

Key words: Reproductive Biology, Reproductive Tract, Sperm, Carboxylesterase, Review

Send Correspondence to: Prof., Dr. Alexander T. Mikhailov, Developmental Biology Unit, Institute of Health Sciences, University of La Coruña, As Xubias s/n, 15006 La Coruña, Spain, Tel.: +34-981-16 70 00, Fax: +34-981- 13 87 14, E-mail: margot@udc.es

This manuscript is available on line at:

http://www.bioscience.org/2000/d/mikhail/fulltext.htm