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REVIEW

# Insect hemolymph clotting

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**Abstract** The clot's appearance in different large-bodied insects has been described, but until recently, little was known about any insect clot's molecular makeup, and few experiments could directly test its function. Techniques have been developed in *Drosophila* (fruit fly) larvae to identify clotting factors that can then be tested for effects on hemostasis, healing, and immunity. This has revealed unanticipated complexity in the hemostatic mechanisms in these larvae. While the clot's molecular structure is not yet fully understood, progress is being made, and the loss of clotting factors has been shown to cause subtle immune defects. The few similarities between coagulation in different insect species and life stages, and the current state of knowledge about coagulation in insects are discussed.

**Keywords** Insect coagulation · Insect immunity · Wound healing · Phenoloxidase · *Drosophila* · *Galleria*

## Introduction

Coagulation is the formation of an insoluble matrix in the blood or hemolymph that stops bleeding (hemostasis), assists wound healing, and protects against infection. Coagulation has long interested scientists. The closed circulatory systems of vertebrates necessitate tight control over coagulation since thromboses can cause ischemic damage and death. In contrast, the open circulatory systems of insects permit coagulation to be more exuberant to quickly seal wounds, limit fluid loss, restore the hydrostatic skeletons of soft bodied animals, and entrap microbes at wound sites. The coagulation of insect hemolymph was reviewed in this journal 7 years ago [1]. Considerable progress has been made since then, particularly in the model insect *Drosophila melanogaster*. This review begins with a summary of work that has been done in a wide variety of insects, followed by a description of more recent studies. Few similarities have been found between coagulation in different species, and differences have been found between species and between different life stages within species. This begs the question how coagulation evolved in insects under different environmental, physiological, and immunological pressures.

We are still far from molecular understanding of coagulation in any insect. However, the identification of clotting factor genes in *Drosophila* bring the fly's powerful genomic and molecular genetic tools into play for dissecting the process of coagulation and its effects on wound healing and immune defense.

## Coagulation is caused by interaction of hemocyte and plasma factors

Over 30 years ago, a comprehensive review of coagulation cited over 100 studies in 1,600 insect species extending as far back as 1891 [2]. Much of this work described the patterns of hemocyte behavior and the appearance of the clot in light and phase microscopy. Four patterns of coagulation were described based on the microscopic appearance of hemocytes and their interaction with plasma factors [3].

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Light and electron microscope studies on larval hemolymph from *Galleria mellonella* (greater wax moth) showed changes in the granules of larval hemocytes and exocytosis ex vivo, suggesting that granular contents were involved in coagulation [4]. In rare in vivo studies of wounded *Galleria mellonella* larvae, bits of fatbody cells were drawn into the wound with granulocytes that subsequently degranulated, resulting in the formation of a matrix to which hemocytes attached [5, 6]. Hemocytes are also required for coagulation in locust larvae. Separation of hemocytes and plasma blocked coagulation in *Locusta migratoria* (migratory locust) plasma, which could then be induced by readdition of hemocytes [7]. Similar results were found in *Leucophaea maderae* (Madeira cockroach) larvae [8]. The involvement of both hemocytes and plasma factors appears to be a general feature of coagulation in insects.

The study of insect coagulation has long been complicated by the complexity of classifying different types of hemocytes in insects and the relationships between hemocyte types in different species, (reviewed in [8]). Readers are also referred to reviews on the classification of insect hemocytes and studies on their involvement in coagulation and immune reactions [9–19].

## Biochemistry

Blocking hemocyte and plasma activation is an important step in the study of coagulation. Anticoagulants also reveal chemical aspects of clot formation. Acids, heat, cold, and ultrasonic waves were shown to prevent coagulation in the hemolymph of different insects, and 33 vertebrate anticoagulants were tested on 14 species of insects with varying degrees of effectiveness [2]. This demonstrated that the molecular mechanisms of insect coagulation are different from the coagulation of vertebrate blood. However, these tests of anticoagulants were not coupled with molecular and biochemical analyses of coagulation in these insects.

One common feature of many of these studies in different insect species is the requirement for $Ca^{2+}$ in hemolymph coagulation. Chelating $Ca^{2+}$ blocked coagulation in larvae of cockroaches [8], *Galleria* [20], and *Drosophila* [21]. While cockroach larval coagulation is inhibited by $Ca^{2+}$ chelators, it was not affected by protease inhibitors. Thus, $Ca^{2+}$ is unlikely to be required for a protease cascade like that of vertebrate coagulation. Instead, the inhibition of coagulation by iodoacetamide (a cysteine blocker that would also inhibit protein cross-linking) in *Leucophaea* larval hemolymph [22] suggested that $Ca^{2+}$ is required for a protein cross-linking enzyme like transglutaminase [8]. In *Drosophila* larvae, candidate clotting factors were identified as transglutaminase substrates [23], and chemical inhibitors or RNAi knockdown of the transglutaminase gene interfered with coagulation [24]. Transglutaminase appears to be a common coagulation factor because it is involved in coagulation in other arthropods as well as acting as vertebrate clotting factor XIIIa [25–31]. Arthropod coagulation has been particularly well characterized in horseshoe crabs. *Tachypleus* (Japanese horseshoe crab) coagulogen contains a cystine knot like vertebrate nerve growth factor and *Drosophila* protein Spaetzle [32]. The *Limulus* (Atlantic horseshoe crab) coagulogen is also structurally similar to Spaetzle, suggesting divergent evolution of coagulation in horseshoe crabs and other arthropods [33].

Lipophorin also appears to be a common insect clotting factor. Lipophorin-like lipoproteins have been identified as coagulogens in noninsect arthropods [26, 34–36]. Biochemical studies on hemolymph from larger-bodied insects showed that lipophorin levels were reduced in post-coagulation serum in *Locusta migratoria* [37–41] and *Galleria mellonella* larvae, where apolipoproteins were identified in precipitates from cell-free hemolymph [42]. Loss of lipophorin in post-coagulation serum was also found for the cockroaches *Periplaneta americana* (American cockroach) and *Leucophaea maderae*, and the incorporation of lipophorin in cockroach clots was shown directly by immunoelectrophoresis and thin layer chromatography [8, 43]. A hemolymph lipoprotein was shown to be involved in melanization in *Tenebrio molitor* (yellow mealworm) [44], consistent with lipophorin involvement in the clot and its subsequent melanization. Apolipophorins I and II were identified as candidate clotting factors in *Drosophila* larval hemolymph [21, 23]. A fine silk-like paracrystalline array of particles that appeared similar to apolipoprotein was observed in negative stain electron micrographs of hemolymph from *Anopheles gambiae* (malaria mosquito) larvae, and this together with identification of apolipophorin 1 by pull-out (see below) suggested lipophorin's presence in the mosquito clot [45].

## Molecular genetics

The rapidity of hemolymph coagulation makes biochemical study of clotting factors difficult. Lipophorins were shown to be involved in coagulation in the larvae of cockroaches and *Galleria* as described above. Antibodies generated against a precipitate induced in cell-free *Galleria* larval hemolymph recognized phenoloxidase, hexamerin (arylphorin), lipophorin, hexamerin, and an α-crystallin/small heat shock protein [20]. This was an innovative approach to directly identify clotting factors, but the induction of precipitate by adding rabbit red blood cells to cell-free larval hemolymph was somewhat artificial, and

the involvement of these proteins in coagulation in *Galleria* must still be confirmed by other means.

*Drosophila melanogaster* is the insect model system with the most molecular genetic tools for testing gene involvement in biological processes, but it was not considered a good model for clotting studies because of its small size and because clotting had never been observed in adults. The development of larval experimental techniques skirted these obstacles. Drawing out a strand from coagulating *Drosophila* larval hemolymph with a fine needle demonstrated that coagulation occurred, and provided a measure of clot strength and stiffness [21, 46]. The *Drosophila* larval clot was visualized directly by scanning electron microscopy and shown to resemble the *Galleria* larval clot [46]. Bleeding larval hemolymph onto paramagnetic Dynabeads (Dynal Corporation/Invitrogen) caused the beads to aggregate, and this was taken as another measure of coagulation. Magnetic isolation of the beads (pull-out) and subsequent elution of bound proteins directly identified them as candidate clotting factors [21]. Clotting factors were also indirectly identified by proteomic comparison of plasma and serum after using pull-out to remove clot proteins from larval serum [23].

Surprisingly, the panel of genes coding for candidate clotting factors in *Drosophila* did not appear to include a clear set of evolutionarily conserved coagulogen genes with homologs in the sequenced genomes of other insects [21, 23]. The first clotting factors tested by functional genomics were *fondue* (*fon*) and *hemolectin* (*hml*). Fondue was isolated both by pull-out [21] and by proteomic comparison of post-clot serum and pre-clot plasma [23]. The *fon* gene is induced by infection with the slow kinetics expected for replenishment of a clotting factor [47]. The novel Fondue protein is a strong transglutaminase substrate [23], and Fondue was confirmed as a clotting factor when RNAi knockdown of *fon* transcription reduced bead aggregation and strand draw-out and affected wound healing. Knockdown of *fon* also caused a pupal cuticle phenotype, suggesting that Fondue protein is part of the cuticle as well as the clot [48]. A Fondue-GFP fusion protein makes the larval clot and cuticle fluoresce [24], consistent with the incorporation of Fondue protein in both the clot and the cuticle. Chemical inhibition or RNAi knockdown of transglutaminase also caused increasing ductility and loss of clot strand draw-out, similar to RNAi knockdown of *fon* or another clotting factor, Eig71Ee (also called gp150 and I71-7) [24, 49]. The similarity of these effects confirms the involvement of transglutaminase and its substrates Fondue and Eig761Ee in coagulation in *Drosophila* larvae, even if the interaction between Fondue, Eig761Ee, and other clot proteins is not yet fully understood, and the chemical structure of the clot itself is still unclear. The predicted amino acid sequence of Fondue protein contains multiple repeats with high levels of glycine and alanine (like silk fibroin and other cuticular proteins) and glutamine, making it a good substrate for transglutaminase [48]. These features are not unique, making it difficult to identify homologous genes in other insects. It may be that Fondue was originally a cuticle protein that was appropriated for a role in coagulation in *Drosophila* [24].

Hemolectin is a von Willebrand factor domain-bearing protein expressed by larval hemocytes, and RNAi knockdown of *hml* caused a bleeding defect in larvae [50]. Like Fondue, Hemolectin was isolated both by pull-out [21] and by proteomic comparison of post-clot serum and pre-clot plasma [23]. Loss of Hemolectin protein abolished bead aggregation and strand draw-out, confirming its involvement in *Drosophila* larval coagulation [51]. A novel mutation of the *hml* gene; $hml^{AC35}$, was isolated in a forward genetic screen of *Drosophila* larvae whose hemolymph did not cause bead aggregation, and this was further evidence of Hemolectin's importance in coagulation [51]. Homologs of *hml* are found in other Drosophilids and honeybee *Apis mellifera* (European honeybee) [51], and a gene called *hemocytin* was identified in *Bombyx mori* (silkworm) [52]. Comparing the predicted amino acid sequences of these different proteins did not reveal the C-type lectin domain originally proposed for *hemocytin* (and making "hemolectin" a misnomer), but the two discoidin domains present in these proteins could account for their agglutinating activity [51]. The modular structure of these proteins suggests they could have arisen through a process of exon-shuffling [53], and that coagulation may have evolved in insects by a tinkering process [28]. The evolution of Hemolectin, other clotting factors, and of coagulation itself could be clarified if the functional history of protein domains can be traced in *Drosophila* and other insects.

The clots of large insects first form as a soft, initial, "primary" clot that is subsequently melanized [8]. This is also the case in *Drosophila* larvae, where initial clot formation is unaffected by the loss of phenoloxidase, while later melanization adds strength and makes it more brittle [46]. Coagulation in mosquito larvae may be different. Delicate paracrystalline lipophorin structures detected in *Anopheles gambiae* hemolymph did not form in the presence of phenoloxidase inhibitor phenylthiourea, so phenoloxidase may be involved at an early stage in coagulation in these larvae [45].

Phenoloxidase activation and release have been extensively studied in insects and arthropods (reviewed in [54]). In *Drosophila*, full systemic phenoloxidase activation results from activation of a serine protease cascade and breakdown of serine protease inhibitor Serpin-27A [55]. In adults, this depends on transcriptional induction pathways

that can be activated by microbial factors such as peptidoglycan involving PGRP-LE, *Toll*, and *imd/relish* [47, 55–60]. Recently, Serpin-28D was also shown to regulate hemolymph phenoloxidase in larvae and adults [61]. Crystal cells, a class of hemocytes, are the source hemolymph phenoloxidase [14, 62]. Activation of crystal cells and phenoloxidase at the larval clot do not depend on gene transcription pathways triggered by peptidoglycan or other microbial products. Instead, clot melanization can be triggered by endogenous signals, such as negatively charged inner plasma membrane phospholipids like phosphatidylserine. The JNK pathway, small GTPases, and TNF homolog Eiger were all required for the induction and rupture of crystal cells at the larval clot [63].

## Wound healing

Coagulation contributes to wound healing. Even though repair of the epithelium and cuticle continue after the clot has formed and bleeding has stopped, the clot may serve as a scaffold for repair. This was shown in *Galleria* larvae where hemocytes attached to the melanized clot on the hemocoel-side and helped form the basal lamina, after which the clot was pushed out of the healing wound [6]. Coagulation and wound healing both depend on hemocytes. Hemocytes participate in coagulation and are found associated with the clot, and they are important for wound healing. Hemocyte chemotaxis to wound sites has been shown in *Drosophila* embryos [64, 65]. Although coagulation has not been demonstrated in embryos, it is possible that hemocyte chemotaxis contributes to both processes. The clot attracts hemocytes and may provide further cues that promote or modulate wound healing. Coagulation and wound healing also appear to depend on many of the same molecular processes in *Drosophila*. The JNK pathway and small GTPases are involved in wound healing (reviewed in [66] and references therein), [67]. They are also required for crystal cell activation and the melanization of the clot [63]. Fondue protein was found in both the cuticle and the clot [48], and phenoloxidase activity is required for wound healing [20, 68, 69] as well as for clot maturation [6, 46, 63]. In *Anopheles* mosquito larvae, phenoloxidase may play a greater role in coagulation than in *Drosophila* larvae [45], so in the mosquito, links between coagulation and wound healing may be even stronger.

## Immune function

In horseshoe crabs, clots kill bound bacteria [70–72]. Bacteria injected into *Galleria mellonella* and *Pieris brassicae* (large white cabbage butterfly) larvae were caught in a matrix (nodule) formed by hemocyte degranulation [73]. *Galleria* bacterial entrapping nodules were melanized and consolidated in a process similar to coagulation [5]. Bacteria added to *Galleria mellonella* larval hemolymph ex vivo were not phagocytized, but were caught up in the clot matrix [4, 46]. Bacteria were similarly entrapped in the *Drosophila* larval clot [46, 51]. Bactericidal activity was not demonstrated for the *Drosophila* clot [46]. However, whether bacteria are killed in insect clots or merely entrapped and pushed outside the hemocoel with the clot as in *Galleria*, this still leaves open the possibility of a clot function in immune defense.

In adult *Periplaneta americana* cockroaches, injected particles were concentrated at the wound site, and the immune response as measured by phenoloxidase activity was higher there than elsewhere in the animal [74]. Phenoloxidase has long been accepted as an important part of insect immunity (full consideration of the large number of these studies is beyond the scope of this review, and readers are referred to [75]). This was recently complicated by the report that mutant *Drosophila* adults lacking a prophenoloxidase activating enzyme that showed no hemolymph melanization or cleavage of prophenoloxidase zymogen were no more susceptible than wild-type to infection by single Gram (+) or Gram (−) bacterial species or fungus [58]. In contrast, a subsequent study of *Drosophila* adults bearing mutations in a different phenoloxidase pathway protease gene showed a variety of phenotypes in response to infection by seven different bacteria and fungi [76].

The disagreement between these studies shows how the study of immune defenses in insects and in *Drosophila* in particular is becoming increasingly complex. Studying coagulation's role in immunity is no less so. Loss of *hml* and RNAi knock-down of *fondue* resulted in greater sensitivity to infection in adults, but this was only detectable in genetically sensitized backgrounds [48, 51]. Assuming some form of coagulation in *Drosophila* adults, these results are consistent with a clot function in immune defense.

## Role of nucleic acids

Vertebrate lymphocytes produce DNA-containing extracellular "nets" or "traps" that bind and kill bacteria [77–79]. These vertebrate nets are similar to the insect clot in appearance, raising the question whether nucleic acids are involved in insect coagulation. No DNA was detected in *Drosophila* larval clots as measured by DAPI staining of *Drosophila* larval clots [46]. In contrast, nucleic acid stain SYTOX Green labeled the *Galleria* larval clot [42]. This difference could reflect differences between the clots in

these Dipteran and Lepidopteran species, or it could be due to differences in procedure or staining sensitivities of the two dyes. Note that it was also reported that bleeding *Galleria* caterpillars into RNA- or DNA-containing solutions increased hemocyte cell adhesion and induced clot formation similar to bleeding into bacteria lipopolysaccharide, while no clot was detectable in hemolymph alone [42]. In contrast, others have reported coagulation in *Galleria* larval hemolymph without microbial factor elicitors [1, 8]. Additional experiments are required to confirm the procoagulant activity of nucleic acids and the presence of DNA in the *Galleria* larval clot, and if this is a shared feature of coagulation in *Drosophila* and other insects.

Nucleic acids were also reported to affect immune responsiveness. Injecting *Galleria* RNA alone did not increase the levels of antibacterial activity in *Galleria* larval hemolymph, but injecting *Galleria* RNA in combination with heat-killed *P. luminescens* bacteria significantly increased antibacterial activity as measured by inhibition zone assay. Adding RNA or DNA to injections of *Photorabdus* bacteria increased *Galleria* caterpillar survival [42]. Although these results do not demonstrate antimicrobial activity by the clot specifically, if nucleic acids are part of the *Galleria* larval clot, they could increase the clot's immune activity.

## Life stage differences

Insects with hard cuticles may have hemolymph under less than atmospheric pressure. Thus, they do not bleed noticeably when wounded, so coagulation is not necessary for hemostasis [2]. It has been reported that coagulation does not occur in the adults of species with hard cuticles or in the larvae of some species [80, 81]. However, lack of evidence for coagulation is not proof of its absence. Coagulation has functional importance beyond hemostasis, such as contributing to wound healing and preventing infection. Thus, it seems likely that some form of coagulation also happens in these animals. For example, although no recognizable clot structure has been detected in *Drosophila* adults, loss of genes coding for larval coagulation factors *fondue* [48] and *hemolectin* [51] caused subtle adult immune defects. Fondue protein was found in the adult cuticle as well as in the larval cuticle and clot [24, 48]. Loss of Fondue protein could thus impede adult wound healing, and this might account for the greater *fon* mutant adult sensitivity to injury and infection. However, Hemolectin protein has not been demonstrated in the cuticle, so wound healing defects do not explain the immune sensitivity of *hml* adults. It seems simplest to assume that one or both of these proteins are involved in some form of coagulation in *Drosophila* adults.

It is also possible that coagulation has not been detected in some insects/stages because the assays used were not appropriate or sufficiently sensitive. *Anopheles gambiae* larvae are similar in size to *Drosophila* larvae and survive wounding with similar melanotic scars, so it was assumed that coagulation would also be similar. Yet, it was not possible to draw out strands from *Anopheles* larval hemolymph or detect bead aggregation, the two gross indicators of coagulation developed for *Drosophila* larvae [46]. Nonetheless, very fine paracrystalline structures that may represent coagulation were detected by negative stain electron microscopy, and apolipophorin I and phenoloxidase, two likely clotting factor proteins, were isolated by pull-out from *Anopheles* larval hemolymph [45]. It is likely that other clot structures will be discovered in other insect life stages and species.

## Complexity, redundancy, and future prospects

With so little known about the molecular basis of coagulation in insects, it is premature to present models for a hypothetical generic insect clot that may not exist in reality. The first images of what may be a mosquito larval clot [45] look very different from larval clots in *Drosophila* and *Galleria* [46]. There does not seem to be a body of clearly identifiable, highly conserved clotting factors in published insect genomes. Yet the interaction of hemocyte and plasma factors appears to be a general feature of coagulation in insects, and transglutaminase, lipophorin, and phenoloxidase appear to be common insect coagulation factors. Currently, most is known molecularly about coagulation in *Drosophila* larvae. Although the interactions of identified coagulation factors in the *Drosophila* larval clot are not yet fully clear, a model of the *Drosophila* larval clot is presented to illustrate current knowledge in Fig. 1. Here, unknown signals trigger hemocyte degranulation, upon which hemocyte factors including Hemolectin and Eig71Ee, and plasma factors including Fondue and Lipophorin interact and are acted on by transglutaminase in the presence of $Ca^{2+}$ to produce a primary soft clot. While loss of Hemolectin protein abolished bead aggregation, and strands could not be drawn out of *hml* mutant larval hemolymph, *hml* larvae survived wounding as well as wild-type controls [51]. Similar results were obtained with *fondue* mutant larvae [48]. These results demonstrate the presence of redundant hemostatic mechanisms in larvae, and these are indicated by dashed lines in Fig. 1. The primary clot is later cross-linked and melanized by phenoloxidase to produce the stronger, mature clot.

Little is known about the additional hemostatic mechanisms uncovered in *lam* and *fon* larvae. If we consider the possibility of a different form of coagulation in adults than

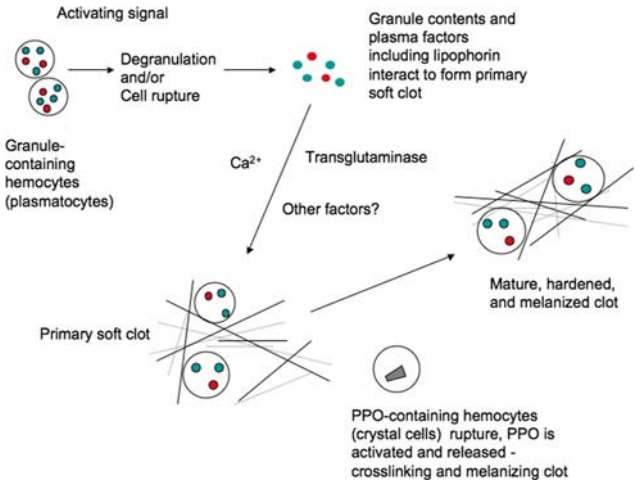


**Fig. 1** Schematic drawing of coagulation in *Drosophila* larvae as it is now understood. Coagulation is initiated when a class of hemocytes—plasmatocytes—are activated to degranulate. The nature of this signal is unknown. Granules containing clotting factors such as Hemolectin and Eig71Ee, and plasma factors such as Fondue are acted on by transglutaminase and possibly other factors in the presence of $Ca^{2+}$ to produce a primary soft clot. Redundant hemostatic mechanisms are indicated by *dashed* and *solid* lines. Additional hemocytes adhere to the clot. Subsequently, crystal cells are activated and rupture to release phenoloxidase, which crosslinks and melanizes the clot to produce its mature, stronger, stiffer, and more brittle form. Not shown are bacteria caught in the clot, or the relation of the clot to the wound, or its involvement in wound healing

in larvae, and accept that larval coagulation factors such as Hemolectin and Fondue have subtle effects on immune defense in the adult, then perhaps the redundant hemostatic mechanisms in the larva and coagulation mechanisms in the adult are related. It is also tempting to speculate that phenoloxidase may do more than melanize the primary soft clot in *Drosophila* larvae. Arguments for a greater hemostatic role for phenoloxidase include the multiplicity of insect phenoloxidase genes with potentially different regulation and function in *Drosophila* as in other insects [1], its known involvement in clot maturation and its activation by denatured lipophorin in *Bombyx* larvae [82], and the greater apparent importance of phenoloxidase in coagulation in *Anopheles* mosquito larvae [45]. The wounding sensitivity of *Bc* mutant *Drosophila* larvae lacking phenoloxidase [51, 83] could indicate reduced clotting as well as defects in wound healing. It also may be that fatbody and tissue fragments are glued together at wound sites to form a plug in *Drosophila* larvae similar to what was observed in *Galleria* larvae [6]. Proteases released by microbes at wounds could also activate or affect phenoloxidase. Exploring these possibilities will require experiments on larvae and adults in vivo, including interactions with natural infections. We now know that a more complete understanding of coagulation in insects and its functions will include appreciation of redundancies in both hemostasis and resistance to infection, and differences between insect stages and species.

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